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1/20, C12R 1:35)(74) Agents: ELDERKIN, Dianne, B. et al.; Woodcock Wash-
burn LLP, 46th floor, One Liberty Place, Philadelphia, PA
19103 (US).

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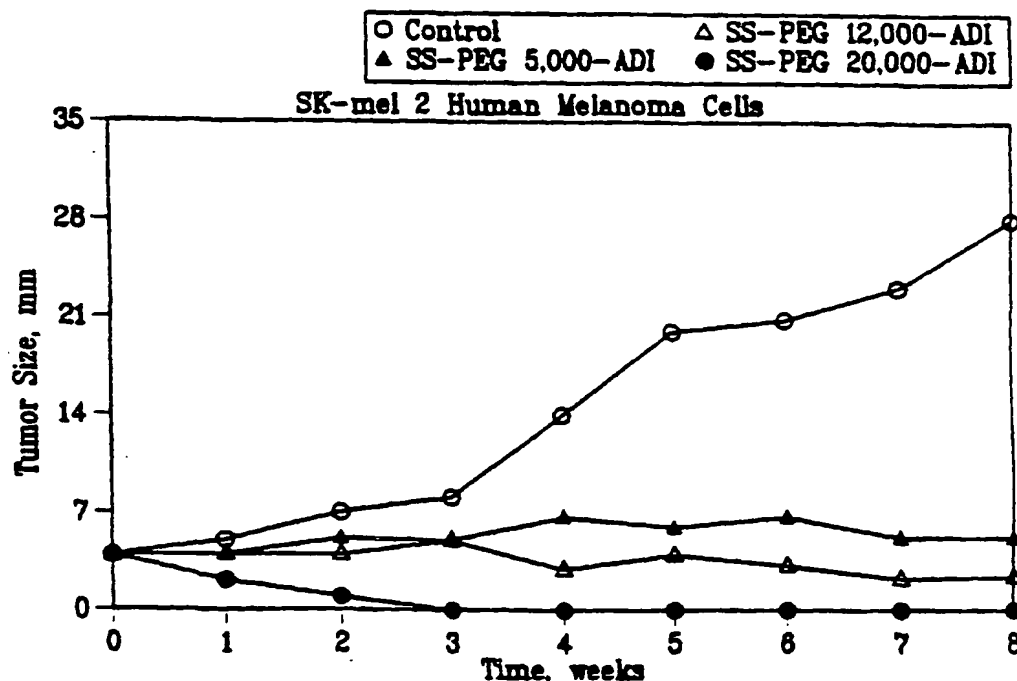
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09/723,546 28 November 2000 (28.11.2000) US(71) Applicant (for all designated States except US):
PHOENIX PHARMACOLOGICS, INC. [US/US];
115 John Robert Thomas Drive, Exton, PA 19341 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): CLARK, Mike, A. [/];
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(54) Title: MODIFIED ARGININE DEIMINASE



(57) Abstract: The present invention is directed to arginine deiminase modified with polyethylene glycol, to methods of treating cancer, and to methods of treating and/or inhibiting metastasis.

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MODIFIED ARGININE DEIMINASE

Related Applications

This application is a continuation in part application of U.S. Patent Application Serial No. 09/023,809, allowed, which claims priority to U.S. Provisional
5 Patent Application Serial No. 60/046,200, filed on May 12, 1997.

Field of the Invention

The present invention is directed to arginine deiminase modified with polyethylene glycol, to methods for treating cancer, and to methods for treating and/or inhibiting metastasis.

10

Background of the Invention

Malignant melanoma (stage 3) and hepatoma are fatal diseases which kill most patients within one year of diagnosis. In the United States, approximately 16,000 people die from these diseases annually. The incidence of melanoma is rapidly increasing in the United States and is even higher in other countries, such as Australia. The incidence
15 of hepatoma, in parts of the world where hepatitis is endemic, is even greater. For example, hepatoma is one of the leading forms of cancer in Japan and Taiwan. Effective treatments for these diseases are urgently needed.

Selective deprivation of essential amino acids has been used to treat some forms of cancer. The best known example is the use of L-asparaginase to lower levels of
20 asparagine as a treatment for acute lymphoblastic leukemia. The L-asparaginase most frequently used is isolated from *E. coli*. However, clinical use of this enzyme is compromised by its inherent antigenicity and short circulating half-life, as described by Y.K. Park, et al, *Anticancer Res.*, 1:373-376 (1981). Covalent modification of *E. coli* L-

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asparaginase with polyethylene glycol reduces its antigenicity and prolongs its circulating half-life, as described, for example, by Park, *Anticancer Res.*, *supra*; Y. Kamisaki et al, *J. Pharmacol. Exp. Ther.*, 216:410-414 (1981); and Y. Kamisaki et al, *Gann.*, 73:47-474 (1982). Although there has been a great deal of effort to identify other essential amino acid degrading enzymes for the treatment of cancer, none have been approved, primarily because deprivation of essential amino acids, by definition, results in numerous, and severe, side effects.

It has been reported that enzymes which degrade non-essential amino acids, such as arginine, may be an effective means of controlling some forms of cancer. For example, arginine deiminase (ADI) isolated from *Pseudomonas putida* was described by J.B. Jones, "The Effect of Arginine Deiminase on Murine Leukemic Lymphoblasts," Ph.D. Dissertation, The University of Oklahoma, pages 1-165 (1981). Although effective in killing tumor cells *in vitro*, ADI isolated from *P. putida* failed to exhibit efficacy *in vivo* because it had little enzyme activity at a neutral pH and was rapidly cleared from the circulation of experimental animals. Arginine deiminase derived from *Mycoplasma arginini* is described, for example, by Takaku et al, *Int. J. Cancer*, 51:244-249 (1992), and U.S. Patent No. 5,474,928, the disclosures of which are hereby incorporated by reference herein in their entirety. However, a problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of arginine deiminase from *Mycoplasma arginini*, via a cyanuric chloride linking group, with polyethylene glycol was described by Takaku et al., *Jpn. J. Cancer Res.*, 84:1195-1200 (1993). However, the modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group.

There is a need for compositions which degrade non-essential amino acids and which do not have the problems associated with the prior art. The present invention is directed to these, as well as other, important ends.

Summary of the Invention

The present invention is directed to arginine deiminase modified with polyethylene glycol. In a preferred embodiment, the arginine deiminase is modified with polyethylene glycol, having a total weight average molecular weight of about 1,000 to about 50,000, directly or through a biocompatible linking group.

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Another embodiment of the invention is directed to methods of treating cancer, including, for example, sarcomas, hepatomas and melanomas. The invention is also directed to methods of treating and/or inhibiting the metastasis of tumor cells.

These and other aspects of the present invention will be elucidated in the following detailed description of the invention.

Brief Description of the Drawings

Figure 1 depicts the amino acid sequences of arginine deiminase cloned from *Mycoplasma arginini* (the top amino acid sequence SEQ ID NO: 1, identified as ADIPROT), *Mycoplasma arthritides* (the middle amino acid sequence SEQ ID NO: 2, identified as ARTADIPRO), and *Mycoplasma hominus* (the bottom amino acid sequence SEQ ID NO: 3, identified as HOMADIPRO).

Figures 2A and 2B are graphs showing the effect of a single dose of native arginine deiminase and arginine deiminase modified with polyethylene glycol (*e.g.*, molecular weight 5,000) on serum arginine levels and serum citrulline levels in mice.

Figure 3 is a graph showing the effects on serum arginine levels when PEG10,000 is covalently bonded to ADI via various linking groups.

Figure 4 is a graph showing the effect that the linking group and the molecular weight of the polyethylene glycol have on citrulline production in mice injected with a single dose of PEG-ADI.

Figures 5A and 5B are graphs showing the dose response that ADI-SS-PEG5,000 had on serum arginine and citrulline levels. **Figures 5C and 5D** are graphs showing the dose response that ADI-SS-PEG20,000 had on serum arginine and citrulline levels.

Figure 6 is a graph showing the antigenicity of native ADI, ADI-SS-PEG5,000, and ADI-SS-PEG20,000.

Figure 7 is a graph showing the effect that treatments with ADI-SS-PEG5,000, ADI-SS-PEG12,000 or ADI-SS-PEG20,000 had on tumor size in mice which were injected with SK-mel 2 human melanoma cells.

Figure 8 is a graph showing the effect that treatments with ADI-PEG20,000 had on tumor size in mice which were injected with SK-mel 28, SK-mel 2 or M24-met human melanoma cells.

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Figure 9 is a graph showing the effect that treatments with ADI-PEG5,000, ADI-PEG12,000 or ADI-PEG20,000 had on the survival of mice which were injected with human hepatoma SK-Hep1 cells.

Figure 10 depicts the amino acid sequences of arginine deiminase cloned from *Streptococcus pyogenes* (the top amino acid sequence SEQ ID NO: 6, identified as STRADIPYR) and *Streptococcus pneumoniae* (the bottom amino acid sequence SEQ ID NO: 7, identified as STRADIPNE).

Figure 11 depicts the amino acid sequences of arginine deiminase cloned from *Borrelia burgdorferi* (the top amino acid sequence SEQ ID NO: 8, identified as BORADIBUR) and *Borrelia afzelii* (the bottom amino acid sequence SEQ ID NO: 9, identified as BORADIAFZ).

Figure 12 depicts the amino acid sequence of *Giardia intestinalis* (the top amino acid sequence SEQ ID NO: 10, identified as QIAADIINT), *Clostridium perfringens* (the middle amino acid sequence SEQ ID NO: 11, identified as CLOADIPER) and *Bacillus licheniformis* (the bottom amino acid sequence SEQ ID NO: 12, identified as BACADILIC).

Figure 13 depicts the amino acid sequence of *Enterococcus faecalis* (the top amino acid sequence SEQ ID NO: 13, identified as ENTADIFAE) and *Lactobacillus sake* (the bottom amino acid sequence SEQ ID NO: 14, identified as LACADISAK).

Detailed Description of the Invention

Normal cells do not require arginine for growth, since they can synthesize arginine from citrulline in a two step process catalyzed by argininosuccinate synthase and argininosuccinate lyase. In contrast, melanomas, hepatomas and some sarcomas do not express argininosuccinate synthase; therefore, they are auxotrophic for arginine. This metabolic difference may be capitalized upon to develop a safe and effective therapy to treat these forms of cancer. Arginine deiminase catalyzes the conversion of arginine to citrulline, and may be used to eliminate arginine. Thus, arginine deiminase may be utilized as a treatment for melanomas, hepatomas and some sarcomas.

Native arginine deiminase may be found in microorganisms and is antigenic and rapidly cleared from circulation in a patient. These problems may be overcome by

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covalently modifying arginine deiminase with polyethylene glycol (PEG). Arginine deiminase covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG." When compared to native arginine deiminase, ADI-PEG retains most of its enzymatic activity, is far less antigenic, has a
5 greatly extended circulating half-life, and is much more efficacious in the treatment of tumors.

"Polyethylene glycol" or "PEG" refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH_2CH_2)_nOH$, wherein n is at least 4. "Polyethylene glycol" or
10 "PEG" is used in combination with a numeric suffix to indicate the approximate weight average molecular weight thereof. For example, PEG5,000 refers to polyethylene glycol having a total weight average molecular weight of about 5,000; PEG12,000 refers to polyethylene glycol having a total weight average molecular weight of about 12,000; and PEG20,000 refers to polyethylene glycol having a total weight average molecular weight
15 of about 20,000.

"Melanoma" may be a malignant or benign tumor arising from the melanocytic system of the skin and other organs, including the oral cavity, esophagus, anal canal, vagina, leptomeninges, and/or the conjunctivae or eye. The term "melanoma" includes, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile
20 melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungual melanoma and superficial spreading melanoma.

"Hepatoma" may be a malignant or benign tumor of the liver, including, for example, hepatocellular carcinoma.

"Patient" refers to an animal, preferably a mammal, more preferably a
25 human.

"Biocompatible" refers to materials or compounds which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic and disease states.

Throughout the present disclosure, the following abbreviations may be
30 used: PEG, polyethylene glycol; ADI, arginine deiminase; SS, succinimidyl succinate;

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SSA, succinimidyl succinamide; SPA, succinimidyl propionate; and NHS, N-hydroxy-succinimide.

The present invention is based on the unexpected discovery that ADI modified with polyethylene glycol provides excellent results in treating certain types of cancer and inhibiting the metastasis of cancer. ADI may be covalently bonded to
5 polyethylene glycol with or without a linking group, although a preferred embodiment utilizes a linking group.

In the present invention, the arginine deiminase gene may be derived, cloned or produced from any source, including, for example, microorganisms, recombinant
10 biotechnology or any combination thereof. For example, arginine deiminase may be cloned from microorganisms of the genera *Mycoplasma*, *Clostridium*, *Bacillus*, *Borrelia*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Qiardia*. It is preferred that arginine deiminase is cloned from *Mycoplasma pneumoniae*, *Mycoplasma hominus*, *Mycoplasma arginini*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, *Borrelia*
15 *afzelii*, *Qiardia intestinalis*, *Clostridium perfringens*, *Bacillus licheniformis*, *Enterococcus faecalis*, *Lactobacillus sake*, or any combination thereof. In particular, the arginine deiminase used in the present invention may have one or more of the amino acid sequences depicted in Figures 1 and 10-13.

In certain embodiments of the present invention, it is preferred that arginine
20 deiminase is cloned from microorganisms of the genus *Mycoplasma*. More preferably, the arginine deiminase is cloned from *Mycoplasma arginini*, *Mycoplasma hominus*, *Mycoplasma arthritides*, or any combination thereof. In particular, the arginine deiminase used in the present invention may have one or more of the amino acid sequences depicted in Figure 1.

25 In one embodiment of the present invention, the polyethylene glycol (PEG) has a total weight average molecular weight of about 1,000 to about 50,000; more preferably from about 3,000 to about 40,000, more preferably from about 5,000 to about 30,000; more preferably from about 8,000 to about 30,000; more preferably from about 11,000 to about 30,000; even more preferably from about 12,000 to about 28,000; still
30 more preferably from about 16,000 to about 24,000; even more preferably from about

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18,000 to about 22,000; even more preferably from about 19,000 to about 21,000, and most preferably about 20,000. Generally, polyethylene glycol with a molecular weight of 30,000 or more is difficult to dissolve, and yields of the formulated product are greatly reduced. The polyethylene glycol may be a branched or straight chain, preferably a
5 straight chain. Generally, increasing the molecular weight of the polyethylene glycol decreases the immunogenicity of the ADI. The polyethylene glycol having a molecular weight described in this embodiment may be used in conjunction with ADI, and, optionally, a biocompatible linking group, to treat cancer, including, for example, melanomas, hepatomas and sarcomas, preferably melanomas.

10 In another embodiment of the present invention, the polyethylene glycol has a total weight average molecular weight of about 1,000 to about 50,000; preferably about 3,000 to about 30,000; more preferably from about 3,000 to about 20,000; more preferably from about 4,000 to about 12,000; still more preferably from about 4,000 to about 10,000; even more preferably from about 4,000 to about 8,000; still more preferably
15 from about 4,000 to about 6,000; with about 5,000 being most preferred. The polyethylene glycol may be a branched or straight chain, preferably a straight chain. The polyethylene glycol having a molecular weight described in this embodiment may be used in conjunction with ADI, and optionally, a biocompatible linking group, to treat cancer, including, for example, melanomas, hepatomas and sarcomas, preferably hepatomas.

20 The linking group used to covalently attach ADI to PEG may be any biocompatible linking group. As discussed above, "biocompatible" indicates that the compound or group is non-toxic and may be utilized *in vitro* or *in vivo* without causing injury, sickness, disease or death. PEG can be bonded to the linking group, for example, via an ether bond, an ester bond, a thiol bond or an amide bond. Suitable biocompatible
25 linking groups include, for example, an ester group, an amide group, an imide group, a carbamate group, a carboxyl group, a hydroxyl group, a carbohydrate, a succinimide group (including, for example, succinimidyl succinate (SS), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), succinimidyl succinamide (SSA) or N-hydroxy succinimide (NHS)), an epoxide group, an oxycarbonylimidazole group (including, for
30 example, carbonyldimidazole (CDI)), a nitro phenyl group (including, for example,

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nitrophenyl carbonate (NPC) or trichlorophenyl carbonate (TPC)), a trysylate group, an aldehyde group, an isocyanate group, a vinylsulfone group, a tyrosine group, a cysteine group, a histidine group or a primary amine. Preferably, the biocompatible linking group is an ester group and/or a succinimide group. More preferably, the linking group is SS, SPA, SCM, SSA or NHS; with SS, SPA or NHS being more preferred, and with SS or SPA being most preferred.

Alternatively, ADI may be coupled directly to PEG (i.e., without a linking group) through an amino group, a sulfhydryl group, a hydroxyl group or a carboxyl group.

ADI may be covalently bonded to PEG, via a biocompatible linking group, using methods known in the art, as described, for example, by Park et al, *Anticancer Res.*, 1:373-376 (1981); and Zaplipsky and Lee, *Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications*, J.M. Harris, ed., Plenum Press, NY, Chapter 21 (1992), the disclosures of which are hereby incorporated by reference herein in their entirety.

The attachment of PEG to ADI increases the circulating half-life of ADI.

Generally, PEG is attached to a primary amine of ADI. Selection of the attachment site of polyethylene glycol on the arginine deiminase is determined by the role of each of the sites within the active domain of the protein, as would be known to the skilled artisan. PEG may be attached to the primary amines of arginine deiminase without substantial loss of enzymatic activity. For example, ADI cloned from *Mycoplasma arginini*, *Mycoplasma arthritides* and *Mycoplasma hominus* has about 17 lysines that may be modified by this procedure. In other words, the 17 lysines are all possible points at which ADI can be attached to PEG via a biocompatible linking group, such as SS, SPA, SCM, SSA and/or NHS. PEG may also be attached to other sites on ADI, as would be apparent to one skilled in the art in view of the present disclosure.

From 1 to about 30 PEG molecules may be covalently bonded to ADI. Preferably, ADI is modified with about 7 to about 15 PEG molecules, more preferably from about 9 to about 12 PEG molecules. In other words, about 30% to about 70% of the primary amino groups in arginine deiminase are modified with PEG, preferably about 40% to about 60%, more preferably about 45% to about 55%, and most preferably about 50% of the primary amino groups in arginine deiminase are modified with PEG. When PEG is covalently bonded to the end terminus of ADI, preferably only 1 PEG molecule is utilized.

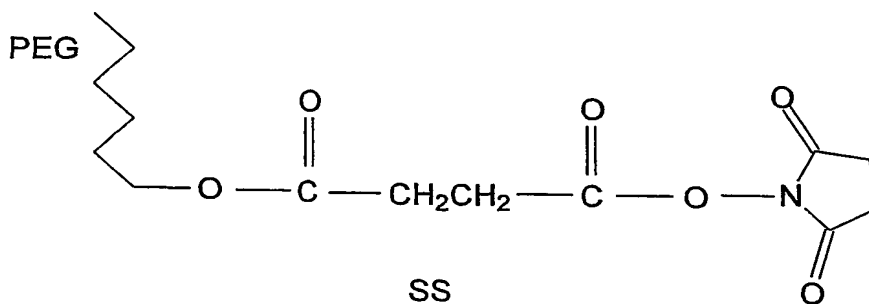
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Increasing the number of PEG units on ADI increases the circulating half life of the enzyme. However, increasing the number of PEG units on ADI decreases the specific activity of the enzyme. Thus, a balance needs to be achieved between the two, as would be apparent to one skilled in the art in view of the present disclosure.

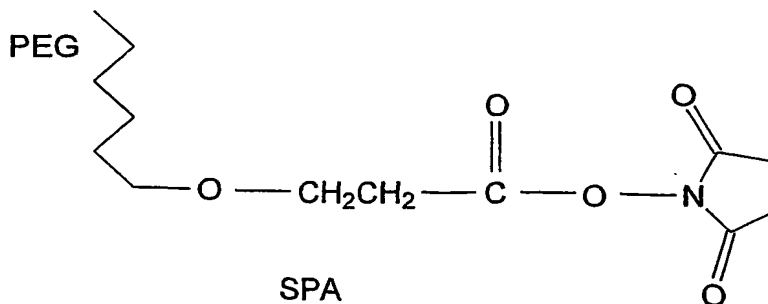
5 In the present invention, a common feature of the most preferred biocompatible linking groups is that they attach to a primary amine of arginine deiminase via a maleimide group. Once coupled with arginine deiminase, SS-PEG has an ester linkage next to the PEG, which may render this site sensitive to serum esterase, which may release PEG from ADI in the body. SPA-PEG and PEG2-NHS do not have an ester
10 linkage, so they are not sensitive to serum esterase.

In the present invention, the particular linking groups do not appear to influence the circulating half-life of PEG-ADI or its specific enzyme activity. However, it is critical to use a biocompatible linking group in the present invention. PEG which is attached to the protein may be either a single chain, as with SS-PEG, SPA-PEG and SC-
15 PEG, or a branched chain of PEG may be used, as with PEG2-NHS. The structural formulas of the preferred linking groups in the present invention are set forth below.

SS-PEG:

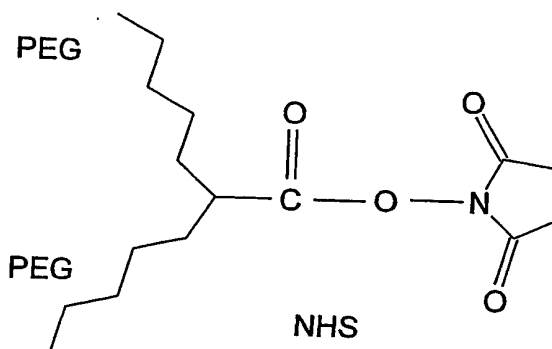


20 SPA-PEG:



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PEG2-NHS:



A therapeutically effective amount of one of the compounds of the present invention is an amount that is effective to inhibit tumor growth. Generally, treatment is initiated with small dosages which can be increased by small increments until the optimum effect under the circumstances is achieved. Generally, a therapeutic dosage of compounds of the present invention may be from about 1 to about 200 mg/kg twice a week to about once every two weeks. For example, the dosage may be about 1 mg/kg once a week as a 2 ml intravenous injection to about 20 mg/kg once every 3 days. The optimum dosage with ADI-SS-PEG5,000 may be about twice a week, while the optimum dosage with ADI-SS-PEG20,000 may be from about once a week to about once every two weeks. PEG-ADI may be mixed with a phosphate buffered saline solution, or any other appropriate solution known to those skilled in the art, prior to injection. The PEG-ADI formulation may be administered as a solid (lyophilate) or as a liquid formulation, as desired.

The methods of the present invention can involve either *in vitro* or *in vivo* applications. In the case of *in vitro* applications, including cell culture applications, the compounds described herein can be added to the cells in cultures and then incubated. The compounds of the present invention may also be used to facilitate the production of monoclonal and/or polyclonal antibodies, using antibody production techniques well known in the art. The monoclonal and/or polyclonal antibodies can then be used in a wide variety of diagnostic applications, as would be apparent to one skilled in the art.

The *in vivo* means of administration of the compounds of the present invention will vary depending upon the intended application. As one skilled in the art will

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recognize, administration of the PEG-ADI composition of the present invention can be carried out, for example, orally, intranasally, intraperitoneally, parenterally, intravenously, intralymphatically, intratumorally, intramuscularly, interstitially, intra-arterially, subcutaneously, intraocularly, intrasynovial, transepithelial, and transdermally.

5

Examples

The invention is further demonstrated in the following examples, which are for purposes of illustration, and are not intended to limit the scope of the present invention.

Example 1: *Production of Recombinant ADI*

Cultures of *Mycoplasma arginini* (ATCC 23243), *Mycoplasma hominus*
10 (ATCC 23114) and *Mycoplasma arthritides* (ATCC 23192) were obtained from the American Type Culture Collection, Rockville, Maryland.

Arginine deiminase was cloned from *Mycoplasma arginini*, *Mycoplasma hominus* and *Mycoplasma arthritides* and expressed in *E. coli* as previously described by S. Misawa et al, *J. Biotechnology*, 36:145-155 (1994), the disclosure of which is hereby
15 incorporated herein by reference in its entirety. The amino acid sequences of arginine deiminase from each of the above species is set forth in Figure 1. The top amino acid sequence, identified as ADIPROT, is from *Mycoplasma arginini*; the middle amino acid sequence, identified as ARTADIPRO, is from *Mycoplasma arthritides*; and the bottom amino acid sequence, identified as HOMADIPRO, is from *Mycoplasma hominus*. Each of
20 the amino acid sequences are more than 96% conserved. Characterization, by methods known to those skilled in the art, of each of the proteins with respect to specific enzyme activity, K_m , V_{max} and pH optima revealed that they were biochemically indistinguishable from each other. The pH optima was determined using a citrate buffer (pH 5-6.5), a phosphate buffer (pH 6.5-7.5) and a borate buffer (pH 7.5-8.5). The K_m and V_{max} were
25 determined by incubating the enzyme with various concentrations of arginine and quantifying citrulline production. The K_m for the various enzymes was about 0.02 to 0.06 μ M and the V_{max} was about 15-20 μ mol/min/mg, the values of which are within standard error of each other.

The arginine deiminase genes were amplified by polymerase chain reaction

using the following primer pair derived from the published sequence of *M. arginini*, as described, for example, by T. Ohno et al, *Infect. Immun.*, 58:3788-3795 (1990), the disclosure of which is hereby incorporated by reference herein in its entirety:

SEQ ID NO: 4, 5'-GGGATCCATGTCTGTATTTGACAGT-3'

5 SEQ ID NO: 5, 5'-TGAAAGCTTTTACTACCACTTAACATCTTTACG-3'

The polymerase chain reaction products were cloned as a Bam HI-Hind III fragment into expression plasmid pQE16. DNA sequence analysis indicated that the fragment derived from *M. arginini* by PCR had the same sequence for the arginine deiminase gene as described by Ohno et al, *Infect. Immun.*, *supra*. The five TGA codons in the ADI gene
10 which encode tryptophan in *Mycoplasma* were changed to TGG codons by oligonucleotide-directed mutagenesis prior to gene expression in *E. coli*, as taught, for example, by J.R. Sayers et al, *Biotechniques*, 13:592-596 (1992). Recombinant ADI was expressed in inclusion bodies at levels of 10% of total cell protein.

The proteins from each of the above three species of *Mycoplasma* have
15 approximately 95% homology and are readily purified by column chromatography. Approximately 200 mg of pure protein may be isolated from 1 liter of fermentation broth. Recombinant ADI is stable for about 2 weeks at 37°C and for at least 8 months when stored at 4°C. As determined by methods known to those skilled in the art, the proteins had a high affinity for arginine (0.04 µM), and a physiological pH optima of about 7.2 to
20 about 7.4.

Example 2: Renaturation and Purification of Recombinant ADI

ADI protein was renatured, with minor modifications, as described by Misawa et al, *J. Biotechnology*, 36:145-155 (1994), the disclosure of which is hereby incorporated herein by reference in its entirety. 100 g of cell paste was resuspended in 800
25 ml of 10 mM K₂PO₄ pH 7.0, 1 mM EDTA (buffer 1) and the cells were disrupted by two passes in a Microfluidizer (Microfluidics Corporation, Newton, MA). Triton X-100 was added to achieve a final concentration of 4% (v/v). The homogenate was stirred for 30 min at 4°C, then centrifuged for 30 min at 13,000 g. The pellet was collected and resuspended in one liter of buffer 1 containing 0.5% Triton X-100. The solution was
30 diafiltered against 5 volumes of denaturation buffer (50 mM Tris HCl, pH 8.5, 10 mM

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DTT) using hollow-fiber cartridges with 100 kD retention rating (Microgon Inc., Laguna Hills, CA). Guanidine HCl was added to achieve a final concentration of 6 M and the solution was stirred for 15 min at 4°C. The solution was diluted 100-fold into refolding buffer 1, 10 mM K₂PO₄, pH 7.0 and stirred for 48 hours at 15°C, particulates were
5 removed by centrifugation at 15,000 x g.

The resulting supernatant was concentrated on a Q Sepharose Fast Flow (Pharmacia Inc., Piscataway, NJ) column preequilibrated in refolding buffer. ADI was eluted using refolding buffer containing 0.2 M NaCl. The purification procedure yielded ADI protein, which was >95% pure as estimated by SDS-PAGE analysis. 8 g of pure
10 renatured ADI protein was produced from 1 kg of cell paste which corresponds to 200 mg purified ADI per liter of fermentation.

ADI activity was determined by micro-modification of the method described by Oginsky et al, *Meth. Enzymol.*, (1957) 3:639-642. 10 µl samples in 0.1 M Na₂PO₄, pH 7.0 (BUN assay buffer) were placed in a 96 well microliter plate, 40 µl of 0.5
15 mM arginine in BUN assay buffer was added, and the plate was covered and incubated at 37°C for 15 minutes. 20 µl of complete BUN reagent (Sigma Diagnostics) was added and the plate was incubated for 10 minutes at 100°C. The plate was then cooled to 22°C and analyzed at 490 nm by a microliter plate reader (Molecular Devices, Inc). 1.0 IU is the amount of enzyme which converts 1 µmole of L-arginine to L-citrulline per minute.
20 Protein concentrations were determined using Pierce Coomassie Blue Protein Assay Reagent (Pierce Co., Rockford, IL) with bovine serum albumin as a standard.

The enzyme activity of the purified ADI preparations was 17-25 IU/mg.

Example 3: Attachment of PEG to ADI

PEG was covalently bonded to ADI in a 100 mM phosphate buffer, pH 7.4.
25 Briefly, ADI in phosphate buffer was mixed with a 100 molar excess of PEG. The reaction was stirred at room temperature for 1 hour, then the mixture was extensively dialyzed to remove unincorporated PEG.

A first experiment was performed where the effect of the linking group used in the PEG-ADI compositions was evaluated. PEG and ADI were covalently bonded
30 via four different linking groups: an ester group or maleimide group, including SS, SSA,

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SPA and SSPA, where the PEG had a total weight average molecular weight of 5,000, 10,000, 12,000, 20,000, 30,000 and 40,000; an epoxy group, PEG-epoxy, where the PEG had a total weight average molecular weight of 5,000; and a branched PEG group, PEG2-NHS, where the PEG had a total weight average molecular weight of 10,000, 20,000 and 40,000.

5.0 IU of the resulting compositions were injected into mice (5 mice in each group). To determine the serum levels of arginine, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for arginine by this method was approximately 2-6 μ M and the reproducibility of measurements within about 8%. The amount of serum arginine was determined by amino acid analysis. As can be seen from the results in Figure 3, the linking group covalently bonding the PEG and ADI did not have an appreciable effect on the ability of ADI to reduce serum arginine *in vivo*. In other words, the linking group may not be critical to the results of the experiment, except that a non-toxic linking group must be used for *in vivo* applications.

A second experiment was performed wherein the effect of the linking group and molecular weight of PEG on serum citrulline levels *in vivo* was evaluated. Mice (5 in each group) were given various compositions of ADI and PEG-ADI in an amount of 5.0 IU. To determine the serum levels of citrulline, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for citrulline by this method was approximately 2-6 μ M and the reproducibility of measurements within about 8%. The amount of citrulline was determined, and the area

- 15 -

under the curve approximated and expressed as $\mu\text{mol days}$.

In Figure 4, the open circles indicate the amount of citrulline produced by native ADI, the filled circles are ADI-SC-PEG, the open squares are ADI-SS-PEG, the open triangles are ADI-SPA-PEG, and the filled triangles are branched chain PEG-NHS-PEG₂. The results in Figure 4 demonstrate that the molecular weight of the PEG determines the effectiveness of the PEG-ADI composition. The effectiveness of the PEG-ADI compositions is not necessarily based on the method or means of attachment of the PEG to ADI, except that a biocompatible linking group must be used for *in vivo* applications.

The results in Figure 4 also demonstrate that the optimal molecular weight of PEG is 20,000. Although PEG30,000 appears to be superior to PEG20,000 in terms of its pharmacodynamics, PEG30,000 is less soluble, which makes it more difficult to work with. The yields, which were based on the recovery of enzyme activity, were about 90% for PEG5,000 and PEG12,000; about 85% for PEG20,000 and about 40% for PEG30,000. Therefore, PEG20,000 is the best compromise between yield and circulating half life, as determined by citrulline production.

In a third experiment, the dose response of serum arginine depletion and the production of citrulline with ADI-SS-PEG5,000 and ADI-SS-PEG20,000 was determined. Mice (5 in each group) were given a single injection of 0.05 IU, 0.5 IU or 5.0 IU of either ADI-SS-PEG5,000 or ADI-SS-PEG20,000. At indicated times, serum was collected, as described above, and an amino acid analysis was performed to quantify serum arginine (Figures 5A and 5C) and serum citrulline (Figures 5B and 5D). Both formulations induced a dose dependent decrease in serum arginine and an increase in serum citrulline. However, the effects induced by ADI-SS-PEG20,000 were more pronounced and of longer duration than the effects induced by ADI-SS-PEG5,000.

Example 4: Selectivity of ADI Mediated Cytotoxicity

The selectivity of arginine deiminase mediated cytotoxicity was demonstrated using a number of human tumors. Specifically, human tumors were tested *in vitro* for sensitivity to ADI-SS-PEG5,000 (50 ng/ml). Viability of cultures was determined after 7 days. For a culture to be defined as "inhibited," greater than 95% of the

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cells must take up Trypan blue dye. A host of normal cells were also tested, including endothelial cells, smooth muscle cells, epithelial cells and fibroblasts, and none were inhibited by ADI-SS-PEG5,000. Although arginine deiminase has no appreciable toxicity towards normal, and most tumor cells, ADI-SS-PEG5,000 greatly inhibited all human
 5 melanomas and hepatomas that were commercially available from the ATCC, MSKCC and Europe.

Table 1: Specificity of Arginine Deiminase Cytotoxicity

Tumor Type	Number of Tumors Tested	Tumors inhibited (%)
Brain	16	0
Colon	34	0
Bladder	3	0
Breast	12	0
Kidney	5	0
Sarcoma	11	64
Hepatoma	17	100
Melanoma	37	100

In a parallel set of experiments, mRNA was isolated from the tumors. Northern blot analyses, using the human argininosuccinate synthase cDNA probe, indicated complete concordance between the sensitivity to arginine deiminase treatment
 20 and an inability to express argininosuccinate synthase. This data suggests that ADI toxicity results from an inability to induce argininosuccinate synthase. Therefore, these cells cannot synthesize arginine from citrulline, and are unable to synthesize the proteins necessary for growth.

Example 5: *Circulating Half-Life*

25 Balb C mice (5 in each group) were injected intravenously with a single 5.0 IU dose of either native arginine deiminase or various formulations of arginine deiminase modified with polyethylene glycol, as indicated in Figures 2A and 2B. To determine the

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serum levels of arginine and citrulline, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then
5 analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for arginine by this method was approximately 6 pM and the reproducibility of measurements within about 8%.

A dose dependent decrease in serum arginine levels, as shown by the solid
10 circles in Figure 2A, and a rise in serum citrulline, as shown by the open triangles in Figure 2B, were detected from the single dose administration of native ADI (filled circles) or ADI-SS-PEG (open triangles). However, the decrease in serum arginine and rise in serum citrulline was short lived, and soon returned to normal. The half life of arginine depletion is summarized in the Table below.

15 **Table 2: Half-Life of Serum Arginine Depletion**

Compound	Half-Life in Days
Native ADI	1
ADI-SS-PEG5,000	5
ADI-SS-PEG12,000	15
ADI-SS-PEG20,000	20
ADI-SS-PEG30,000	22

20

This experiment demonstrates that normal cells and tissues are able to convert the citrulline back into arginine intracellularly while melanomas and hepatomas cannot because they lack argininosuccinate synthetase.

25 **Example 6: Antigenicity of PEG modified ADI**

To determine the antigenicity of native ADI, ADI-SS-PEG5,000, and ADI-

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SS-PEG20,000, the procedures described in, for example, Park, *Anticancer Res.*, *supra*, and Kamisaki, *J. Pharmacol. Exp. Ther.*, *supra*, were followed.. Briefly, Balb C mice (5 in each group) were intravenously injected weekly for 12 weeks with approximately 0.5 IU (100 µg of protein) of native ADI, ADI-SS-PEG5,000 or ADI-SS-PEG20,000. The animals were bled (0.05 ml) from the retro orbital plexus at the beginning of the experiment and at weeks 4, 8 and 12. The serum was isolated and stored at -70°C. The titers of anti-ADI IgG were determined by ELISA. 50 µg of ADI was added to each well of a 96 well micro-titer plate and was incubated at room temperature for 4 hours. The plates were rinsed with PBS and then coated with bovine serum albumin (1 mg/ml) to block nonspecific protein binding sites, and stored over night at 4°C. The next day serum from the mice was diluted and added to the wells. After 1 hour the plates were rinsed with PBS and rabbit anti-mouse IgG coupled to peroxidase was added to the wells. The plates were incubated for 30 min and then the resulting UV absorbance was measured using a micro-titer plate reader. The titer was defined as the highest dilution of the serum which resulted in a two-fold increase from background absorbance (approximately 0.50 OD).

The results are shown in Figure 6. The open circles represent the data obtained from animals injected with native ADI, which was very antigenic. The filled circles represent the data obtained from the animals injected with ADI-SS-PEG5,000, while the open triangles represent the data obtained from the animals injected with ADI-SS-PEG20,000. As can be seen from Figure 6, ADI-SS-PEG5,000 and ADI-SS-PEG20,000 are significantly less antigenic than native ADI. For example, as few as 4 injections of native ADI resulted in a titer of about 10^6 , while 4 injections of any of the PEG-ADI formulations failed to produce any measurable antibody. However, after 8 injections, the ADI-PEG5,000 had a titer of about 10^2 , while ADI-PEG20,000 did not induce this much of an immune response until after 12 injections. The results demonstrate that attaching PEG to ADI blunts the immune response to the protein.

Example 7: Tumor Inhibition of Human Melanomas

The effect of PEG-ADI on the growth of human melanoma (SK-Mel 28) in nude mice was determined. Nude mice (5 in each group) were injected with 10^6 SK-mel 2

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human melanoma cells which were allowed to grow until the tumors reached a diameter of about 3-5 mm. The mice were left untreated (open circles) or were treated once a week for 8 weeks with 5.0 IU of ADI-SS-PEG5,000 (filled triangles), ADI-SS-PEG12,000 (open triangles) or ADI-SS-PEG20,000 (filled circles). The tumor size was measured weekly, and the mean diameter of the tumors is presented in Figure 7.

Figure 8 shows the effectiveness of ADI-SS-PEG20,000 on three human melanomas (SK-mel 2, SK-mel 28, M24-met) grown *in vivo* in nude mice. Nude mice (5 in each group) were injected with 10^6 SK-mel 2, SK-mel 28 or M24-met human melanoma cells. The tumors were allowed to grow until they were approximately 3-5 mm in diameter. Thereafter, the animals were injected once a week with 5.0 IU of ADI-SS-PEG20,000. The results are shown in Figure 8, and show that PEG-ADI inhibited tumor growth and that eventually the tumors began to regress and disappear. Because the tumors did not have argininosuccinate synthetase, they were unable to synthesize proteins (because ADI eliminated arginine and the tumors could not make it) so that the cells "starved to death."

Since M24-met human melanoma is highly metastatic, the animals injected with M24-met human melanoma cells were sacrificed after 4 weeks of treatment and the number of metastases in the lungs of the animals was determined. The control animals had an average of 32 metastases, while the animals treated with ADI-SS-PEG20,000 did not have any metastases. The results appear to indicate that ADI-SS-PEG20,000 not only inhibited the growth of the primary melanoma tumor, but also inhibited the formation of metastases.

It is of interest to note that in over 200 animals tested, the average number of metastases in the control group was 49 ± 18 , while only a single metastasis was observed in 1 treated animal.

Example 8: Tumor Inhibition of Human Hepatomas

The ability of PEG-ADI to inhibit the growth of a human hepatoma *in vivo* was tested. Nude mice (5 in each group) were injected with 10^6 human hepatoma SK-Hep1 cells. The tumors were allowed to grow for two weeks and then the animals were treated once a week with 5.0 IU of SS-PEG5,000-ADI (solid circles), SS-PEG12,000-ADI

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(solid triangles) or SS-PEG20,000-ADI (open triangles). The results are set forth in Figure 9. The untreated animals (open circles) all died within 3 weeks. In contrast, animals treated with ADI had a far longer life expectancy, as can be seen from Figure 9. All the surviving mice were euthanized after 6 months, and necropsy indicated that they were free of tumors.

Surprisingly, PEG5,000-ADI is most effective in inhibiting hepatoma growth *in vivo*. The exact mechanism by which this occurs is unknown. Without being bound to any theory of the invention works, it appears that proteins formulated with SS-PEG5,000-ADI become sequestered in the liver. Larger molecular weights of PEG do not, which may be due to the uniqueness of the hepatic endothelium and the spaces (fenestrae) being of such a size that larger molecular weights of PEG-ADI conjugates are excluded.

Example 9: Application to Humans

PEG5,000-ADI and PEG20,000-ADI were incubated *ex vivo* with normal human serum and the effects on arginine concentration was determined by amino acid analysis, where the enzyme was found to be fully active and capable of degrading all the detectable arginine with the same kinetics as in the experiments involving mice. The reaction was conducted at a volume of 0.1 ml in a time of 1 hour at 37°C.

Additionally, the levels of arginine and citrulline in human serum are identical with that found in mice. PEG-proteins circulate longer in humans than they do in mice. For example, the circulating half life of PEG conjugated adenosine deiminase, asparaginase, glucocerbroidase, uricase, hemoglobulin and superoxide dismutase all have a circulating half life that is 5 to 10 times longer than the same formulations in mice. What this has meant in the past is that the human dose is most often 1/5 to 1/10 of that used in mice. Accordingly, PEG-ADI should circulate even longer in humans than it does in mice.

Each of the patents, patent applications and publications described herein are hereby incorporated by reference herein in their entirety.

Various modifications of the invention, in addition to those described herein, will be apparent to one skilled in the art in view of the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

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What is claimed is:

1. A compound comprising arginine deiminase covalently bonded via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.
2. The compound of claim 1, wherein said linking group is a succinimide group.
3. The compound of claim 2, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate, succinimidyl carboxymethylate, succinimidyl succinamide, N-hydroxy succinimide or combinations thereof.
4. The compound of claim 3, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate or combinations thereof.
5. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.
6. The compound of claim 5, wherein said microorganism is selected from the group consisting of *Mycoplasma arginini*, *Mycoplasma hominus*, *Mycoplasma arthritides* and combinations thereof.
7. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Streptococcus*.
8. The compound of claim 7, wherein said microorganism is selected from the group consisting of *Streptococcus pyogenes*, *Streptococcus pneumoniae* and combinations thereof.

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9. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Borrelia*.

10. The compound of claim 9, wherein said microorganism is selected from the group consisting of *Borrelia burgdorferi*, *Borrelia afzelii*, and combinations thereof.

11. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Qiardia*.

12. The compound of claim 11, wherein said microorganism is *Qiardia intestinalis*.

13. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Clostridium*.

14. The compound of claim 13, wherein said microorganism is *Clostridium perfringens*.

15. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Enterococcus*.

16. The compound of claim 15, wherein said microorganism is *Enterococcus faecalis*.

17. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Lactobacillus*.

18. The compound of claim 17, wherein said microorganism is *Lactobacillus sake*.

19. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Bacillus*.

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20. The compound of claim 19, wherein said microorganism is *Bacillus licheniformis*.

21. The compound of claim 1, wherein said microorganism is selected from the group consisting of *Mycoplasma pneumoniae*, *Mycoplasma hominus*, *Mycoplasma arginini*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Borrelia burgdorferi*, *Borrelia afzelii*, *Giardia intestinalis*, *Clostridium perfringens*, *Bacillus licheniformis*, *Enterococcus faecalis*, *Lactobacillus sake*, and combinations thereof.

22. The compound of claim 1, wherein said arginine deiminase is covalently bonded to about 7 to about 15 polyethylene glycol molecules.

23. The compound of claim 22, wherein said arginine deiminase is covalently bonded to about 9 to about 12 polyethylene glycol molecules.

24. The compound of claim 1, wherein said polyethylene glycol has a total weight average molecular weight of from about 10,000 to about 30,000.

25. A method of enhancing the circulating half life of arginine deiminase comprising modifying said arginine deiminase by covalently bonding said arginine deiminase via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.

26. A method of enhancing the tumoricidal activity of arginine deiminase comprising modifying said arginine deiminase by covalently bonding said arginine deiminase via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.

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27. A method of treating a tumor in a patient comprising administering to said patient the compound of Claim 1.

28. The method of claim 27, wherein said tumor is a melanoma.

29. The method of claim 28, wherein said polyethylene glycol has a total weight average molecular weight of about 20,000

30. The method of claim 28, wherein said linking group is a succinimide group.

31. The method of claim 30, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate, succinimidyl carboxymethylate, succinimidyl succinamide, N-hydroxy succinimide or combinations thereof.

32. The method of claim 27, wherein said tumor is a hepatoma.

33. The method of claim 32, wherein said polyethylene glycol has a total weight average molecular weight of about 5,000

34. The method of claim 32, wherein said linking group is a succinimide group.

35. The method of claim 34, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate, succinimidyl carboxymethylate, succinimidyl succinamide, N-hydroxy succinimide or combinations thereof.

36. The method of claim 27, wherein said tumor is a sarcoma.

37. A method of treating and inhibiting metastases in a patient comprising administering to said patient the compound of claim 1.

1/10

The alignment was done on 3 Protein sequences.

Character to show that a position in the alignment is perfectly conserved: '*'

Character to show that a position is well conserved: '.'

Alignment

ADIPROT	MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLFSAILE	50
ARTADIPRO	MSVFDSKFKGIHVYSEIGELESVLVHEPGREIDYITPARLDELLFSAILE	50
HOMADIPRO	MSVFDSKFNGIHVYSEIGELETVLVHEPGREIDYITPARLDELLFSAILE	50

ADIPROT	SHDARKEHKQFVAELKANDINVVELIDLVAETYDLASQEAQDKLIEEFLE	100
ARTADIPRO	SHDARKEQSQFVAAILKANDINVETIDLVAETYDLASQEAQDKRLIEEFLE	100
HOMADIPRO	SHDARKEHQSFVKIMKDRGINVVELTDLVAETYDLASKAAKEEFIETFE	100
	***** . ** . . . *****	
ADIPROT	DSEPVLSEEHKVVVRNFLKAKKTSRKLVEIMMAGITKYDLGIEADHELIV	150
ARTADIPRO	DSEPVLSEAHKKVVVRNFLKAKKTSRKLVELMMAGITKYDLGVEADHELIV	150
HOMADIPRO	ETVPVLTEANKKAVRAFLLSKPT-HEMVEFMMSGITKYELGVESENELIV	149
	.. *** . . . * * * . . . * *	
ADIPROT	DPMPNLYFTRDPFASVGNVTIHYMRYKVRQRETLSRFVFSNHPKLINT	200
ARTADIPRO	DPMPNLYFTRDPFASVGNVTIHFMYKVRRETLSRFVFRNHPKLINT	200
HOMADIPRO	DPMPNLYFTRDPFASVGNVTIHFMYIVRRRETLSRFVFRNHPKLINT	199

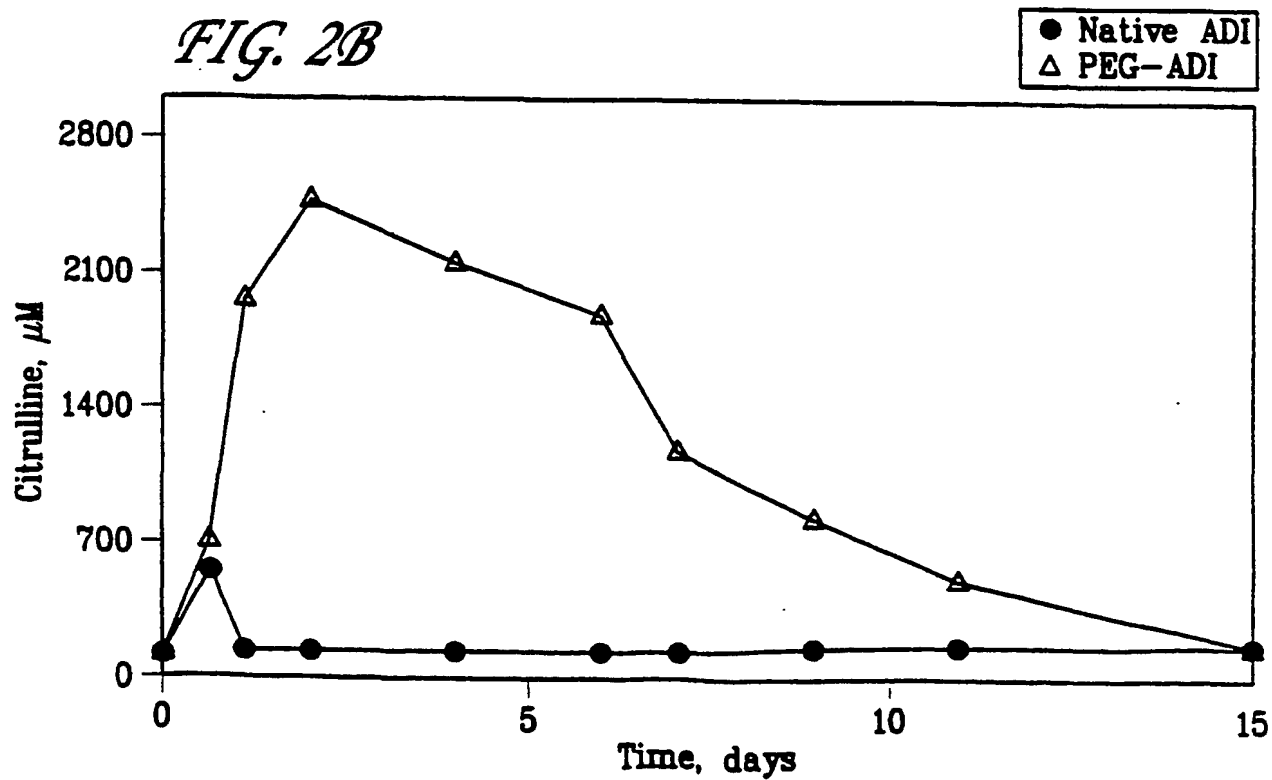
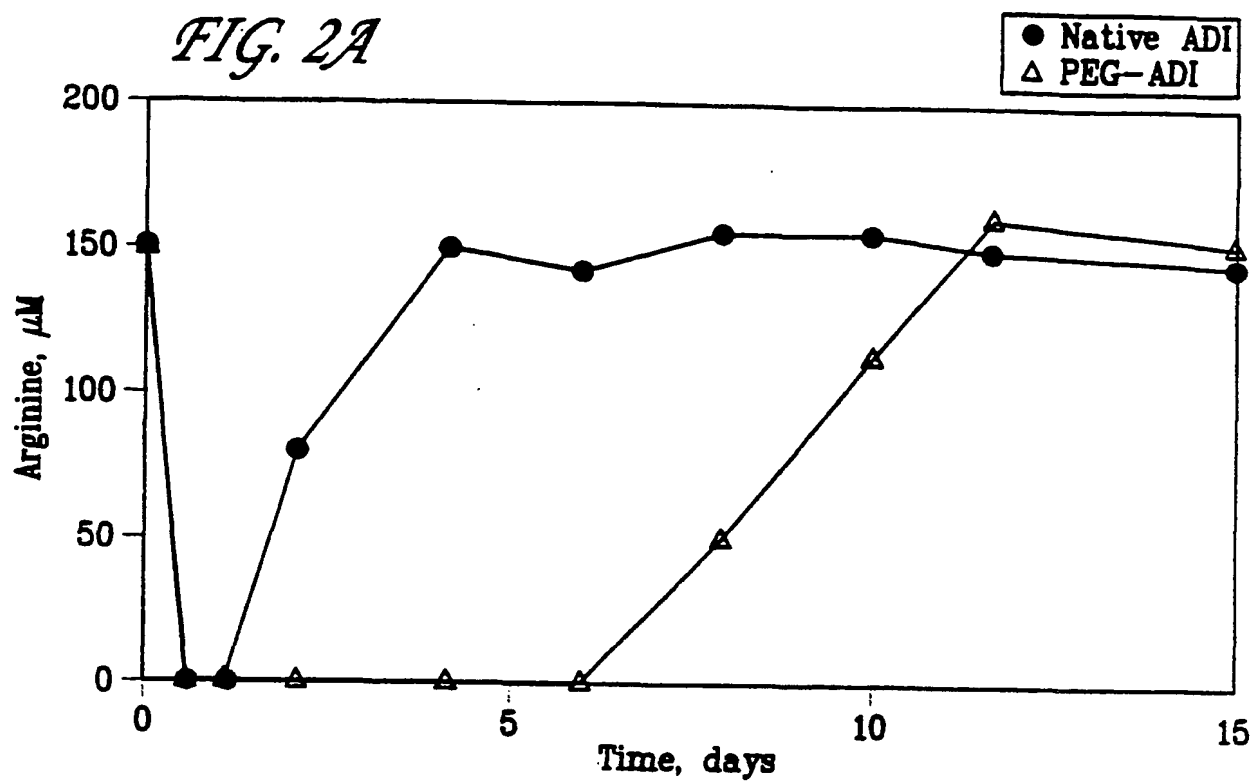
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ARTADIPRO	PWYYDPAMKLSEGGDVFIYNNDTLVGVSERTDLDTVTLLAKNLVANKE	250
HOMADIPRO	PWYYDPAMKMPIEGGDVFIYNNETLVGVSERTDLDTITLLAKNIKANKE	249
	***** ***** * *****	
ADIPROT	CEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV	300
ARTADIPRO	CEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV	300
HOMADIPRO	VEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV	299

ADIPROT	NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDG	350
ARTADIPRO	NGGAEPQPVENGLPLEGLLQSIINKKPVLIPIAGEGASQMEIERETHFDG	350
HOMADIPRO	NGGAEPQPOLNGLPLDKLLASIINKEPVLIPIGGAGATEMEIARETNFDG	349
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ARTADIPRO	TNYLAIRPGVVIGYSRNEKTNAALKAAGIKVLPFHGNQLSLGMGNARCMS	400
HOMADIPRO	TNYLAIRPGVVIGYSRNEKTNAALKAAGITVLPFHGNQLSLGMGNARCMS	399
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ARTADIPRO	MPLSRKDVKW	410
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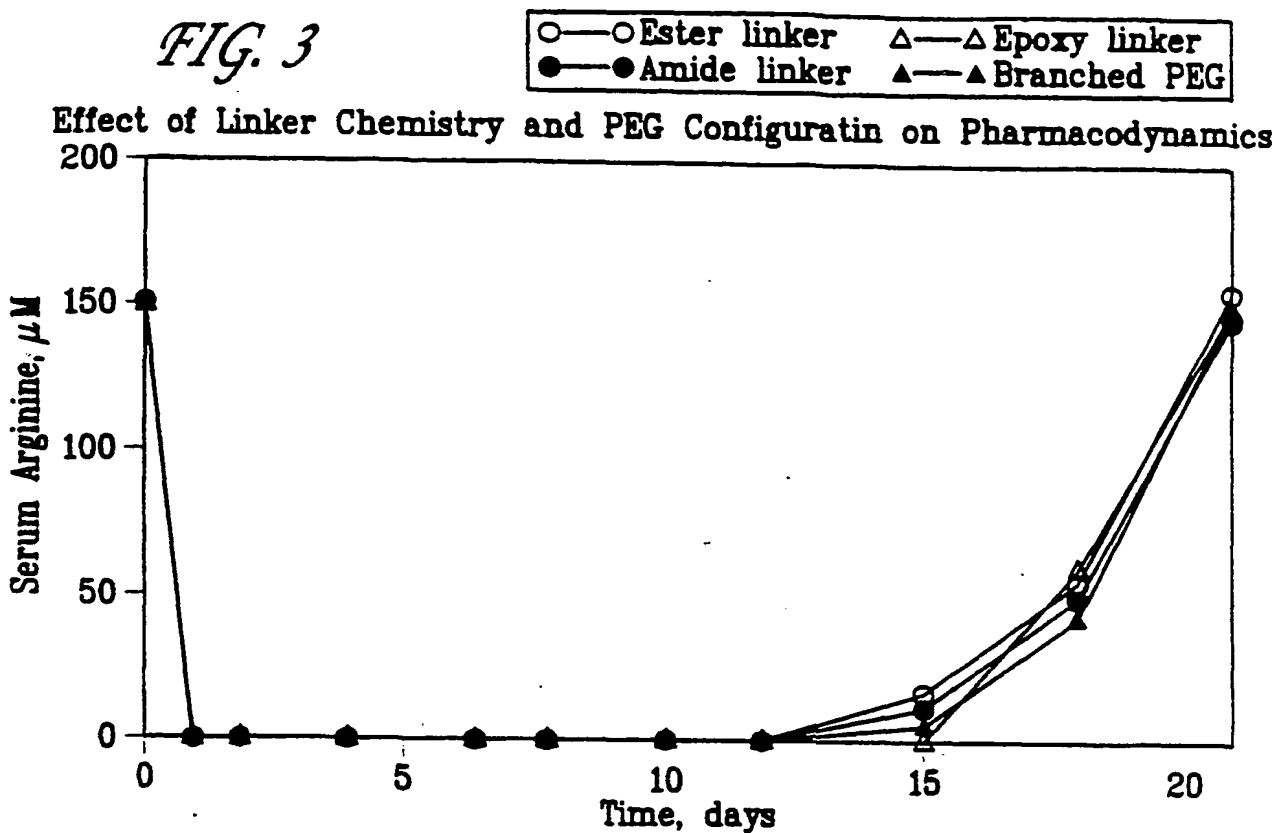
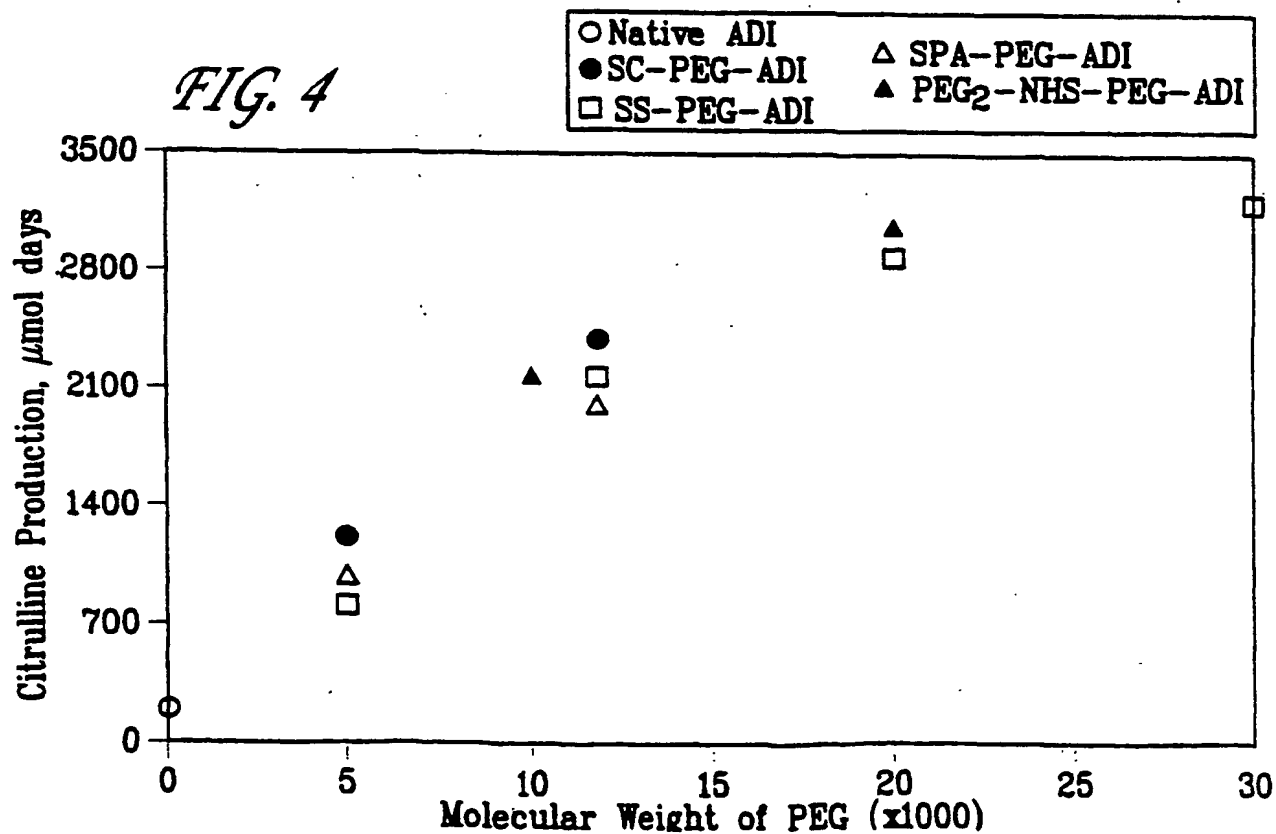
FIG. 1

ADIPROT	=	Mycoplasma arginini
ARTADIPRO	=	Mycoplasma arthritides
HOMADIPRO	=	Mycoplasma hominus

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FIG. 3*FIG. 4*

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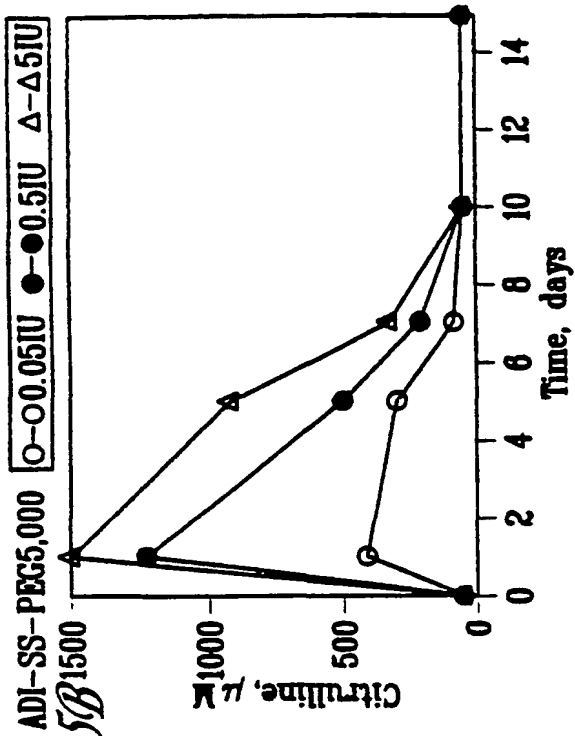


FIG. 5B

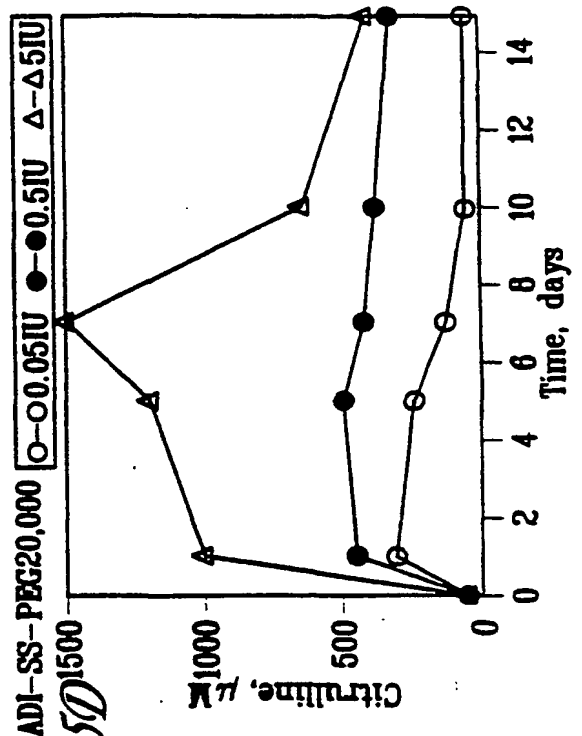


FIG. 5D

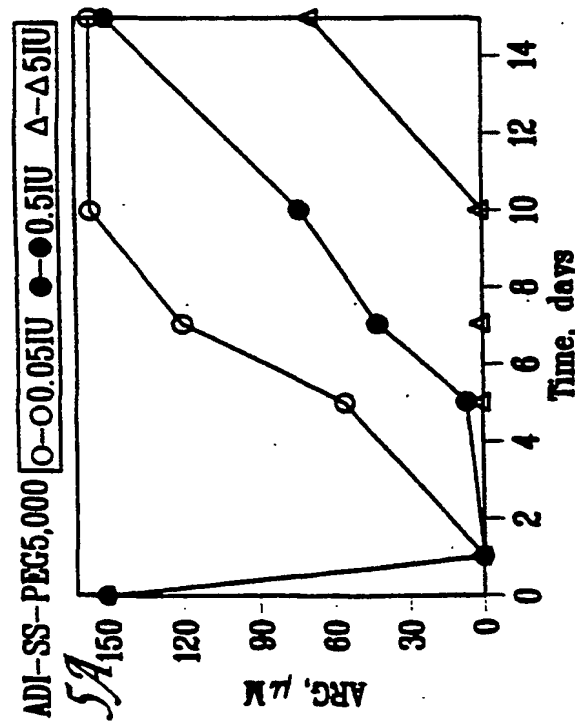


FIG. 5A

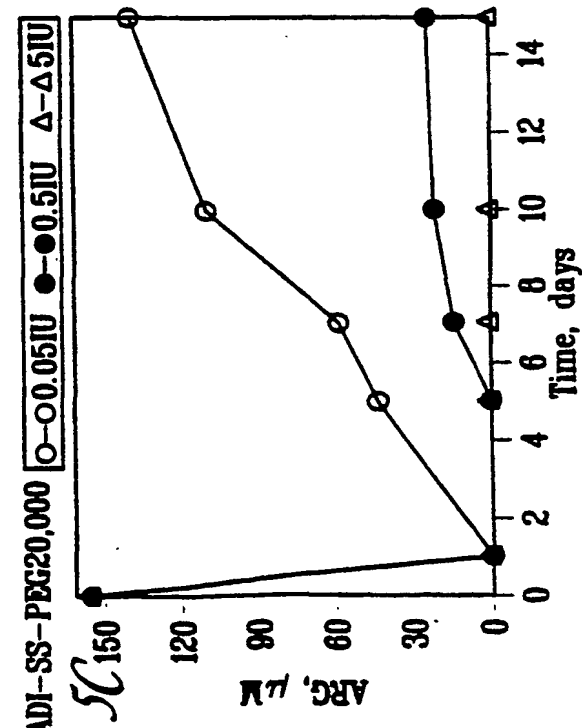


FIG. 5C

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FIG. 6

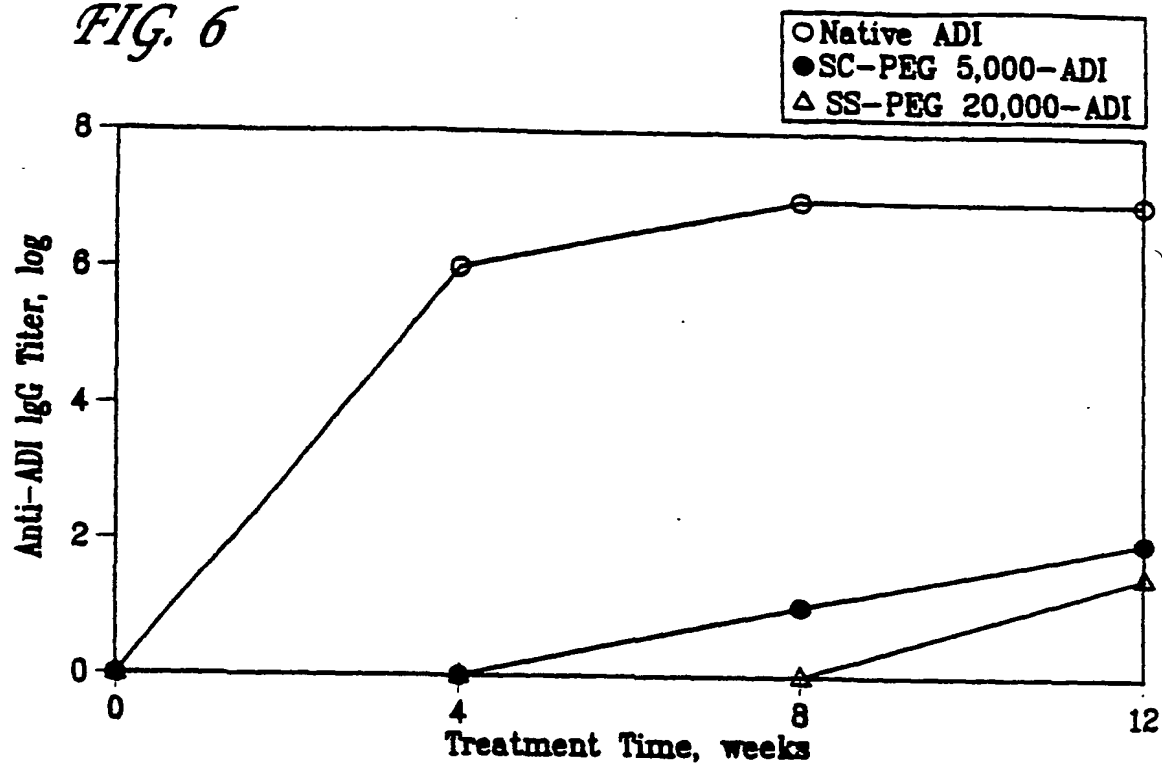
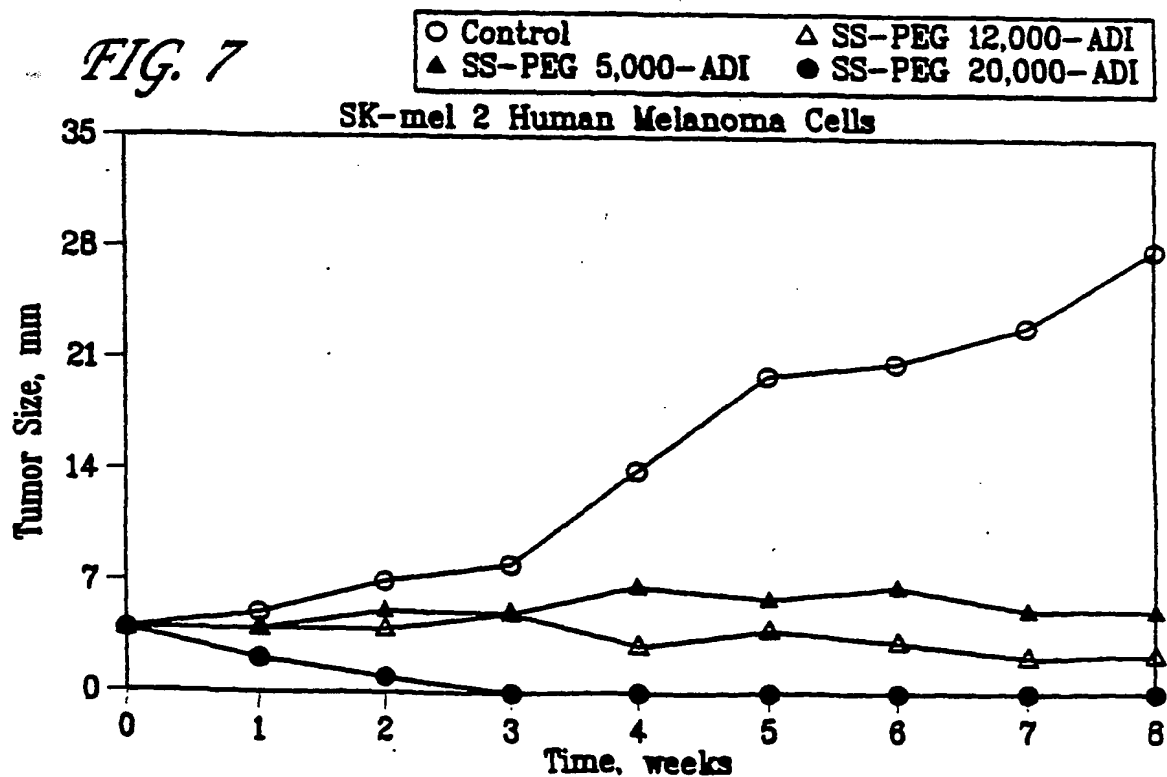
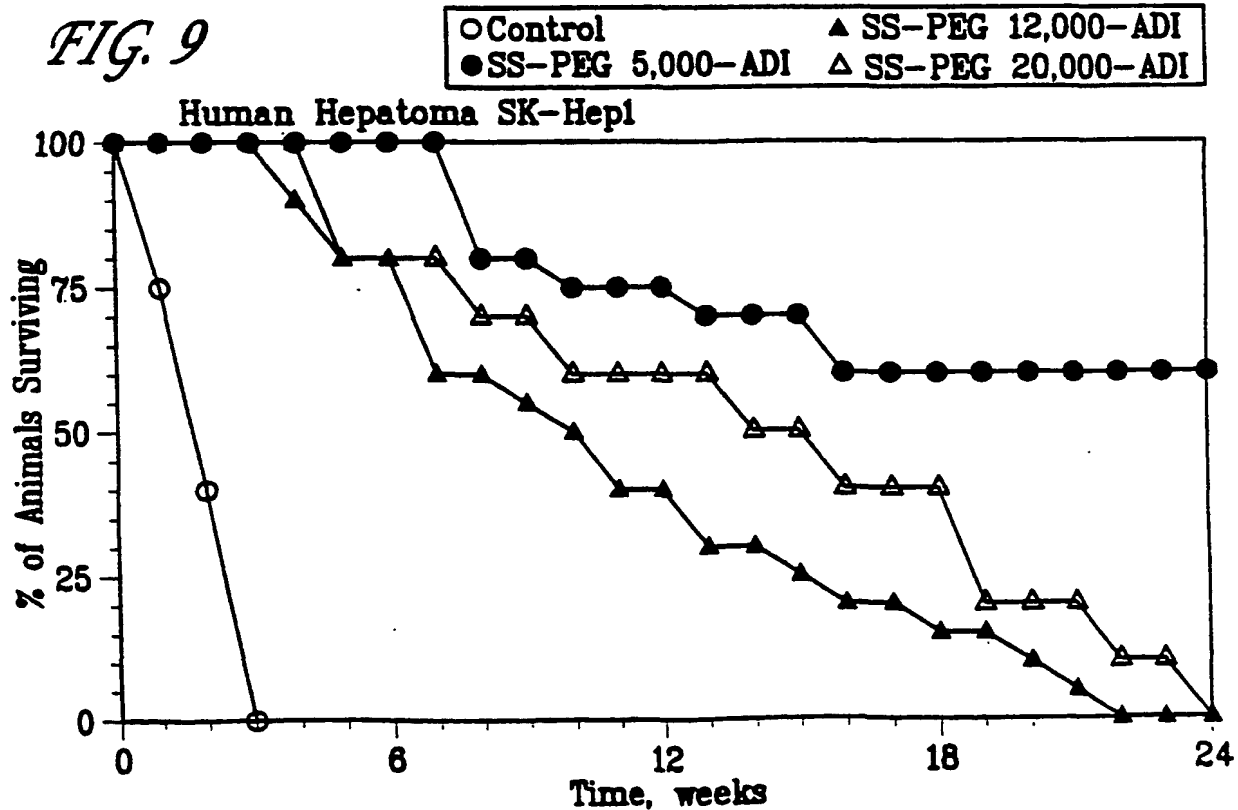
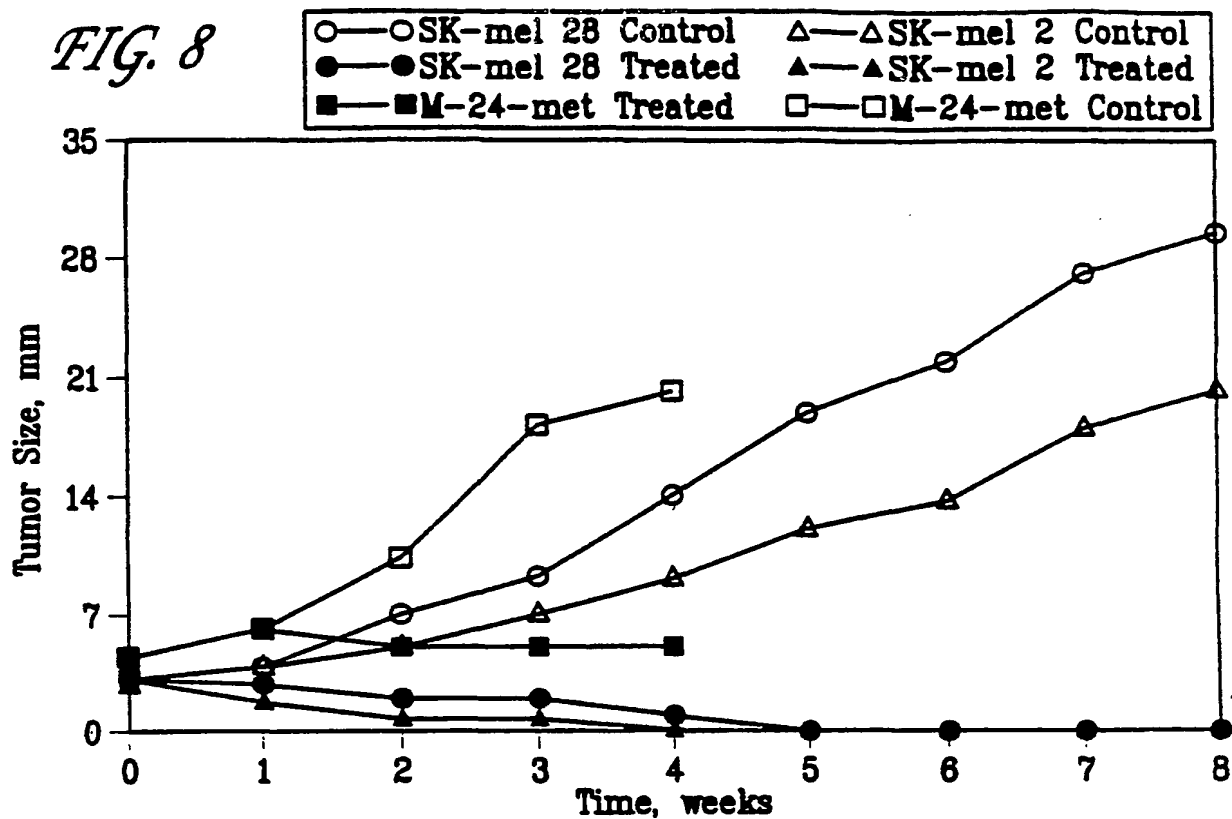


FIG. 7



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7/10

The alignment was done on 2 amino acid sequences.

Alignment

STRADIPYR	MTAQTPIHVYSEIGKLKKVLLHRPGKEIENLMPDYLERLLFDDIPFLEDA	50
STRADIPNE	MSSHPIQVFSEIGKLKKVMLHRPGKELENLLPDYLERLLFDDIPFLEDAQ	50
STRADIPYR	QKEHDAFAQALRDEGIEVLYLETLAAESLVTPEIREAFIDEYLSEANIRG	100
STRADIPNE	KEHDAFAQALRDEGIEVLYLEQLAAESLTSPEIRDQFIEEYLDEANIRDR	100
STRADIPYR	RATKKAIRELLMAIEDNQELIEKTMAGVQKSELPEIPASEKGLTDLVESN	150
STRADIPNE	QTKVAIRELLHGIKDNQELVEKTMAGIQKVELPEIPDEAKDLTDLVESEY	150
STRADIPYR	YPFAIDPMPNLYFTRDPFATIGTGVSLSNHMFSETRNRETLYGKYIFTHHP	200
STRADIPNE	PFAIDPMPNLYFTRDPFATIGNAVSLNHMFADTRNRETLYGKYIFKYHPI	200
STRADIPYR	IYGGGKVPVMVYDRNETTRIEGGDELVL SKDVLAVGISQRTDAASIEKLLV	250
STRADIPNE	YGGKVDLVYNREEDTRIEGGDELVL SKDVLAVGISQRTDAASIEKLLVNI	250
STRADIPYR	NIFKQNLGFKKVLAFEFANNRKFMHLDTVFTMVDYDKFTIHPEIEGDLRV	300
STRADIPNE	FKKNVGFKKVLAFEFANNRKFMHLDTVFTMVDYDKFTIHPEIEGDLHVYS	300
STRADIPYR	YSVTYDNEELHIVEEKGD LAELLAANLGVEKVDLIRCGGDNLVAAGREQW	350
STRADIPNE	VTYENEKLKIVEEKGD LAELLAQNLGVEKVHLIRCGGGNIVAAAREQWND	350
STRADIPYR	NDGSNTLTIAPGVVVVYNRNTITNAILES KGLKLIKIHGSELVRGRGGPR	400
STRADIPNE	GSNTLTIAPGVVVVYDRNTVTNKILEEYGLRLIKIRGSELVRGRGGPRCM	400
STRADIPYR	CMSMPFEREDI	411
STRADIPNE	SMPFEREEV	409

FIG. 10

STRADIPYR = *Streptococcus pyogenes*

STRADIPNE = *Streptococcus pneumoniae*

8/10

The alignment was done on 2 amino acid sequences.

Alignment

BORADIBUR	MEEEYLNPNIFSEIGRLKKVLLHRPGELENLTPLIMKNFLFDDIPYLK	50
BORADIAFZ	MEEYLNPNIFSEIGRLKKVLLHRPGELENLTPFIMKNFLFDDIPYLEV	50
BORADIBUR	VARQEHEVFNILKDNSVEIEYVEDLVSEVLASSVALKNKFISQFILEAE	100
BORADIAFZ	ARQEHEVFASILKNNLVEIEYIEDLISEVLVSSVALENKFISQFILEAEI	100
BORADIBUR	IKTDGVINILKDYFSNLTVDNMVSKMISGVAREELKDCEFSLDDWVNGSS	150
BORADIAFZ	KTDFTINLLKDYFSSLTIDNMISKMISGVVTEELKNYTSSLDDLNGANL	150
BORADIBUR	FVIDPMPNVLFTRDPFASIGNGITINKMYTKVRRRETIFAEYIFKYHSAY	200
BORADIAFZ	FIIDPMPNVLFTRDPFASIGNGVITINKMFTKVRQRETIFAEYIFKYHPVY	200
BORADIBUR	KENVPIWFNRWEETSLEGGDEFVLNKDLLVIGISERTEAGSVEKLAASLF	250
BORADIAFZ	KENVPIWLNRWEEASLEGGDELVLNKGLLVIGISERTEAKSVEKLALSLF	250
BORADIBUR	KNKAPFSTILAFKIPKNRAYMHLDTVFTQIDYSVFTSFTSDDMYFSIYVL	300
BORADIAFZ	KNKTSFDTILAFQIPKNRSYMHLDTVFTQIDYSVFTSFTSDDMYFSIYVL	300
BORADIBUR	TYNSNSNKINIKKEKAKLKDVLSFYLGKIDIIKCAGGDLIHGAREQWND	350
BORADIAFZ	TYNPSSSKIHIKKEKARIKDVLSFYLGKIDIIKCAGGDLIHGAREQWND	350
BORADIBUR	GANVLAIAPGEVIAYSRNHVTNKLFEENGIVHRIIPSELSRGRGGPRCM	400
BORADIAFZ	GANVLAIAPGEIIAYS RNHVTNKLFEENGIVHRIIPSELSRGRGGPRCM	400
BORADIBUR	SMSLVREDI	409
BORADIAFZ	SMPLIREDI	409

FIG. 11

BORADIBUR = *Borrelia burgdorferi*

BORADIAFZ = *Borrelia afzelii*

9/10

The alignment was done on 3 amino acid sequences.

Alignment

QIAADIINT	MTDFSKDKEKLAQATQGGENERAEIVVHLPQGTSFLTSLNPEGNNLEEP	50
CLOADIPER	MRDDRALNVTSEIGRLKTVLLHRPGEEIENLTPDLLDRLLFDDIPYLKVA	50
BACADILIC	MIMTTPIHVYSEIGPLKTVMLKRPGRELENLTPEYLERLLFDDIPFLPAV	50
QIAADIINT	ICPDELRRDHEGFQAVLKEKGCRVYMPYDVLSEASPAEREVLMDQAMASL	100
CLOADIPER	REEHDAFAQTLREAGVEVLYLEVLAEEAIETSDEVKQQFISEFIDEAGVE	100
BACADILIC	QKEHDQFAETLKQQGAEVLYLEKLTAALDDALVREQFIDELLTESKADI	100
QIAADIINT	KYELHATGARITPKMKYCVSDEYKRKVLSALSTRNLVDVILSEPVHILAP	150
CLOADIPER	SERLKEALIEYFNSFSDNKAMVDKMMAGVRKEELKDYHRESLYDQVNNVY	150
BACADILIC	NGAYDRLKEFLLTFDADSMVEQVMGIRKNELEREKKSHLHELMEDHYPE	150
QIAADIINT	GVRNTALVTNSVEIHDSNNMVFMRDQQITTRRGIVMGQFQAPQRRREQVL	200
CLOADIPER	PFVCDPMPNLYFTREPFATIGHGITLNMRTDTRNRETIFAKYIFRHHPR	200
BACADILIC	YLDPMNLYFTRDPAAGSGLTINKMKEPARRESLFMRYIINHHPRFK	200
QIAADIINT	ALIFWKRLGARVVGDCREGGPHCMLEGGDFVPVSPGLAMMGVGLRSTYVG	250
CLOADIPER	FEGKDIPFWFNRNDKTSLEGGDELILSKEILAVGISQRTDSASVEKLAKK	250
BACADILIC	GHEIPVWLDRDFKFNIEGGDELVLNEETVAIGVSERTTAQAIERLVRNLF	250
QIAADIINT	AQYLMKDLLGTRRFVVKDCFDQHQRMDHLDCTFSVLHDKLVVLDYYIC	300
CLOADIPER	LLYYPDTSFKTVLAFKIPVSRAFMHLDTVFTQVDYDKFTVHPGIVGPLEV	300
BACADILIC	QRQSRIRRVLA VEIPKSRAFMHLDTVFTMVD RDQFTIHPAIQGPEGDMRI	300
QIAADIINT	SGMGLRYVDEWIDVGADAVKKAKSSAVTCGNYVLAKANVEFQQWLSSENGY	350
CLOADIPER	YALTKDPENDGQLLVTEEVDTLENILKKYLDRIKLIKCGGGDEIIAARE	350
BACADILIC	FVLERGKTADEIHTTEHNLPVLKRTLGLSDVNLIFCGGGDEIASAREQ	350
QIAADIINT	TTVRIPHEYQLAYGCNNLNLGNNCVLSVHQPTVDFIKADPAYISYCKSNN	400
CLOADIPER	QWNDGSNTLAIAPGEVVVYSRNYVTNEILEKEGIKLHVIPSSSELSRGRGG	400
BACADILIC	WNDGSNTLAIAPGVVVYDRNYISNECLREQGIKVIEIPSGELSRGRGGP	400
QIAADIINT	LPNGLDLVYVPFRGITRMYGSLHCASQVVYRTPLAPAAVKACEQEGDGIA	450
CLOADIPER	PRCMSMPLIREDL	413
BACADILIC	RCMSMPLYREDVK	413
QIAADIINT	AIYEKNGEPVDAAGKKFDCVIYIPSSVDDLIDGLKINLRDDAAPSREIIA	500
QIAADIINT	DAYGLYQKLVSEGRVPYITWRMPSPMPVVS LKGAAGAGSLKAVLDKIPQLT	550
QIAADIINT	PFTPKAVEGAPAAAYTRYLGLEQADICVDIK	580

QIAADIINT = *Giardia intestinalis*
 CLOADIPER = *Clostridium perfringens*
 BACADILIC = *Bacillus licheniformis*

FIG. 12

10/10

The alignment was done on 2 amino acid sequences.

Alignment

ENTADIFAE	MSHPINVSEIGKLKTVMLHRPGKELENLMPDYLERLLFDDIPFLEKAQA	50
LACADISAK	MTSPIHVNSEIGKLKTVLLKRPGEVENITPDIMYRLLFDDIPYLPTIQK	50
ENTADIFAE	EHDFAFAELLRSKDIEVVYLEDLAAEALINEEVRRQFIDQFLEENIRSES	100
LACADISAK	EHDQFAQTLRDNGVEVLYLENLAAEAIDAGDVKEAFLDKMLNESHKSPQ	100
ENTADIFAE	AKEKVRELMLEIDDNEELIQKAIAGIQKQELPKYEQEFLTDMVEADYPFI	150
LACADISAK	VQAALKDYLI SMATLDMVEKIMAGVRTNEIDIKSKALIDVSADDDYPFYM	150
ENTADIFAE	IDPMPNLYFTRDNFATMGHGISLNHMYSVTRQRETIFGQYIFDYHPRFAG	200
LACADISAK	DPMPNLYFTRDPAASMGDGLTINKMTFEARQRESMFMEVIMQHHPRFANQ	200
ENTADIFAE	KEVPRVYDRSESTRIEGGDELILSKEVVAIGISQRTDAASIEKIARNIFE	250
LACADISAK	GAQVWRDRDHIDRMEGGDELILSDKVLAIGISQRTSAQSIEELAKVLFAN	250
ENTADIFAE	QKLGFKNILAFDIGEHRKFMHLDTVFTMIDYDKFTIHPEIEGGLVVYSIT	300
LACADISAK	HSGFEKILAIKIPHKHAMMHLDTVFTMIDYDKFTIHPIQGAGGMVDTYI	300
ENTADIFAE	EKADGDIQITKEKDTLDNILCKYLHLDNVQLIRCGAGNLTAAREQWNDG	350
LACADISAK	LEPGNNDKITHQTDLEKVLRLDALEVPELTLPCGGGDAVVAPREQWND	350
ENTADIFAE	SNTLAIAPGEVVVYDRNTITNKALEEAGVKLNYIPGSELVRGRGGPRCMS	400
LACADISAK	GSNTLAIAPGVVVTYDRNYVSNENLRQYGIKVIEVPSELSRGRGGPRCM	400
ENTADIFAE	MPLYREDL	408
LACADISAK	SMPLVRRKT	409

FIG. 13ENTADIFAE = *Enterococcus faecalis*LACADISAK = *Lactobacillus sake*

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<151> 1998-02-13

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<151> 2000-11-28

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<213> Mycoplasma arginini

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 20 25 30

Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile
 35 40 45

Leu Glu Ser His Asp Ala Arg Lys Glu His Lys Gln Phe Val Ala Glu
 50 55 60

Leu Lys Ala Asn Asp Ile Asn Val Val Glu Leu Ile Asp Leu Val Ala
 65 70 75 80

Glu Thr Tyr Asp Leu Ala Ser Gln Glu Ala Lys Asp Lys Leu Ile Glu
 85 90 95

Glu Phe Leu Glu Asp Ser Glu Pro Val Leu Ser Glu Glu His Lys Val
 100 105 110

Val Val Arg Asn Phe Leu Lys Ala Lys Lys Thr Ser Arg Lys Leu Val
115 120 125

Glu Ile Met Met Ala Gly Ile Thr Lys Tyr Asp Leu Gly Ile Glu Ala
130 135 140

Asp His Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
145 150 155 160

Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Tyr Met Arg
165 170 175

Tyr Lys Val Arg Gln Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Ser
180 185 190

Asn His Pro Lys Leu Ile Asn Thr Pro Trp Tyr Tyr Asp Pro Ser Leu
195 200 205

Lys Leu Ser Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr
210 215 220

Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Gln Thr Val Thr Leu
225 230 235 240

Leu Ala Lys Asn Ile Val Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile
245 250 255

Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr
260 265 270

Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser Pro Ile Ala
275 280 285

Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ala
290 295 300

Glu Pro Gln Pro Val Glu Asn Gly Leu Pro Leu Glu Gly Leu Leu Gln
305 310 315 320

Ser Ile Ile Asn Lys Lys Pro Val Leu Ile Pro Ile Ala Gly Glu Gly
325 330 335

Ala Ser Gln Met Glu Ile Glu Arg Glu Thr His Phe Asp Gly Thr Asn
340 345 350

Tyr Leu Ala Ile Arg Pro Gly Val Val Ile Gly Tyr Ser Arg Asn Glu
355 360 365

Lys Thr Asn Ala Ala Leu Glu Ala Ala Gly Ile Lys Val Leu Pro Phe
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His Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser
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 405 410

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 20 25 30

Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile
 35 40 45

Leu Glu Ser His Asp Ala Arg Lys Glu Gln Ser Gln Phe Val Ala Ile
 50 55 60

Leu Lys Ala Asn Asp Ile Asn Val Val Glu Thr Ile Asp Leu Val Ala
 65 70 75 80

Glu Thr Tyr Asp Leu Ala Ser Gln Glu Ala Lys Asp Arg Leu Ile Glu
 85 90 95

Glu Phe Leu Glu Asp Ser Glu Pro Val Leu Ser Glu Ala His Lys Lys
 100 105 110

Val Val Arg Asn Phe Leu Lys Ala Lys Lys Thr Ser Arg Lys Leu Val
 115 120 125

Glu Leu Met Met Ala Gly Ile Thr Lys Tyr Asp Leu Gly Val Glu Ala
 130 135 140

Asp His Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
 145 150 155 160

Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Phe Met Arg
 165 170 175

Tyr Lys Val Arg Arg Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Arg
 180 185 190
 Asn His Pro Lys Leu Val Asn Thr Pro Trp Tyr Tyr Asp Pro Ala Met
 195 200 205
 Lys Leu Ser Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr
 210 215 220
 Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Asp Thr Val Thr Leu
 225 230 235 240
 Leu Ala Lys Asn Leu Val Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile
 245 250 255
 Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr
 260 265 270
 Trp Leu Thr Met Leu Asp Lys Asn Lys Phe Leu Tyr Ser Pro Ile Ala
 275 280 285
 Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ala
 290 295 300
 Glu Pro Gln Pro Val Glu Asn Gly Leu Pro Leu Glu Lys Leu Leu Gln
 305 310 315 320
 Ser Ile Ile Asn Lys Lys Pro Val Leu Ile Pro Ile Ala Gly Gly Gly
 325 330 335
 Ala Ser Gln Met Glu Ile Glu Arg Glu Thr His Phe Asp Gly Thr Asn
 340 345 350
 Tyr Ile Ala Ile Arg Pro Gly Val Val Ile Gly Tyr Ser Arg Asn Glu
 355 360 365
 Lys Thr Asn Ala Ala Leu Lys Ala Ala Gly Ile Lys Val Leu Pro Phe
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<212> PRT

<213> Mycoplasma hominis

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Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile
      35              40              45

Leu Glu Ser His Asp Ala Arg Lys Glu His Gln Ser Phe Val Lys Ile
      50              55              60

Met Lys Asp Arg Gly Ile Asn Val Val Glu Leu Thr Asp Leu Val Ala
  65              70              75              80

Glu Thr Tyr Asp Leu Ala Ser Lys Ala Ala Lys Glu Glu Phe Ile Glu
      85              90              95

Thr Phe Leu Glu Glu Thr Val Pro Val Leu Thr Glu Ala Asn Lys Lys
      100              105              110

Ala Val Arg Ala Phe Leu Leu Ser Lys Pro Thr His Glu Met Val Glu
      115              120              125

Phe Met Met Ser Gly Ile Thr Lys Tyr Glu Leu Gly Val Glu Ser Glu
      130              135              140

Asn Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg Asp
      145              150              155              160

Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Phe Met Arg Tyr
      165              170              175

Ile Val Arg Arg Arg Glu Thr Leu Phe Ala Arg Phe Val Phe Arg Asn
      180              185              190

His Pro Lys Leu Val Lys Thr Pro Trp Tyr Tyr Asp Pro Ala Met Lys
      195              200              205

Met Pro Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Glu Thr Leu
      210              215              220

Val Val Gly Val Ser Glu Arg Thr Asp Leu Asp Thr Ile Thr Leu Leu

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225	230	235	240
Ala Lys Asn Ile Lys Ala Asn Lys Glu Val Glu Phe Lys Arg Ile Val	245	250	255
Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr Trp	260	265	270
Leu Thr Met Leu Asp Lys Asn Lys Phe Leu Tyr Ser Pro Ile Ala Asn	275	280	285
Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ala Glu	290	295	300
Pro Gln Pro Gln Leu Asn Gly Leu Pro Leu Asp Lys Leu Leu Ala Ser	305	310	315
Ile Ile Asn Lys Glu Pro Val Leu Ile Pro Ile Gly Gly Ala Gly Ala	325	330	335
Thr Glu Met Glu Ile Ala Arg Glu Thr Asn Phe Asp Gly Thr Asn Tyr	340	345	350
Leu Ala Ile Lys Pro Gly Leu Val Ile Gly Tyr Asp Arg Asn Glu Lys	355	360	365
Thr Asn Ala Ala Leu Lys Ala Ala Gly Ile Thr Val Leu Pro Phe His	370	375	380
Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser Met	385	390	395
Pro Leu Ser Arg Lys Asp Val Lys Trp	405		

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<210> 6

<211> 411

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Lys	Lys	Val	Leu	Leu	His	Arg	Pro	Gly	Lys	Glu	Ile	Glu	Asn	Leu	Met
			20					25					30		

Pro	Asp	Tyr	Leu	Glu	Arg	Leu	Leu	Phe	Asp	Asp	Ile	Pro	Phe	Leu	Glu
		35					40					45			

Asp	Ala	Gln	Lys	Glu	His	Asp	Ala	Phe	Ala	Gln	Ala	Leu	Arg	Asp	Glu
	50					55					60				

Gly	Ile	Glu	Val	Leu	Tyr	Leu	Glu	Thr	Leu	Ala	Ala	Glu	Ser	Leu	Val
65					70					75					80

Thr	Pro	Glu	Ile	Arg	Glu	Ala	Phe	Ile	Asp	Glu	Tyr	Leu	Ser	Glu	Ala
				85					90					95	

Asn	Ile	Arg	Gly	Arg	Ala	Thr	Lys	Lys	Ala	Ile	Arg	Glu	Leu	Leu	Met
			100					105					110		

Ala	Ile	Glu	Asp	Asn	Gln	Glu	Leu	Ile	Glu	Lys	Thr	Met	Ala	Gly	Val
		115					120					125			

Gln	Lys	Ser	Glu	Leu	Pro	Glu	Ile	Pro	Ala	Ser	Glu	Lys	Gly	Leu	Thr
	130					135					140				

Asp	Leu	Val	Glu	Ser	Asn	Tyr	Pro	Phe	Ala	Ile	Asp	Pro	Met	Pro	Asn
145					150					155					160

Leu	Tyr	Phe	Thr	Arg	Asp	Pro	Phe	Ala	Thr	Ile	Gly	Thr	Gly	Val	Ser
				165					170					175	

Leu	Asn	His	Met	Phe	Ser	Glu	Thr	Arg	Asn	Arg	Glu	Thr	Leu	Tyr	Gly
			180					185					190		

Lys Tyr Ile Phe Thr His His Pro Ile Tyr Gly Gly Gly Lys Val Pro
 195 200 205
 Met Val Tyr Asp Arg Asn Glu Thr Thr Arg Ile Glu Gly Gly Asp Glu
 210 215 220
 Leu Val Leu Ser Lys Asp Val Leu Ala Val Gly Ile Ser Gln Arg Thr
 225 230 235 240
 Asp Ala Ala Ser Ile Glu Lys Leu Leu Val Asn Ile Phe Lys Gln Asn
 245 250 255
 Leu Gly Phe Lys Lys Val Leu Ala Phe Glu Phe Ala Asn Asn Arg Lys
 260 265 270
 Phe Met His Leu Asp Thr Val Phe Thr Met Val Asp Tyr Asp Lys Phe
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 Thr Ile His Pro Glu Ile Glu Gly Asp Leu Arg Val Tyr Ser Val Thr
 290 295 300
 Tyr Asp Asn Glu Glu Leu His Ile Val Glu Glu Lys Gly Asp Leu Ala
 305 310 315 320
 Glu Leu Leu Ala Ala Asn Leu Gly Val Glu Lys Val Asp Leu Ile Arg
 325 330 335
 Cys Gly Gly Asp Asn Leu Val Ala Ala Gly Arg Glu Gln Trp Asn Asp
 340 345 350
 Gly Ser Asn Thr Leu Thr Ile Ala Pro Gly Val Val Val Val Tyr Asn
 355 360 365
 Arg Asn Thr Ile Thr Asn Ala Ile Leu Glu Ser Lys Gly Leu Lys Leu
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 Ile Lys Ile His Gly Ser Glu Leu Val Arg Gly Arg Gly Gly Pro Arg
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 Cys Met Ser Met Pro Phe Glu Arg Glu Asp Ile
 405 410

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Lys	Val	Met	Leu	His	Arg	Pro	Gly	Lys	Glu	Leu	Glu	Asn	Leu	Leu	Pro
			20					25					30		
Asp	Tyr	Leu	Glu	Arg	Leu	Leu	Phe	Asp	Asp	Ile	Pro	Phe	Leu	Glu	Asp
		35					40					45			
Ala	Gln	Lys	Glu	His	Asp	Ala	Phe	Ala	Gln	Ala	Leu	Arg	Asp	Glu	Gly
	50					55					60				
Ile	Glu	Val	Leu	Tyr	Leu	Glu	Gln	Leu	Ala	Ala	Glu	Ser	Leu	Thr	Ser
65					70					75					80
Pro	Glu	Ile	Arg	Asp	Gln	Phe	Ile	Glu	Glu	Tyr	Leu	Asp	Glu	Ala	Asn
				85					90					95	
Ile	Arg	Asp	Arg	Gln	Thr	Lys	Val	Ala	Ile	Arg	Glu	Leu	Leu	His	Gly
			100					105					110		
Ile	Lys	Asp	Asn	Gln	Glu	Leu	Val	Glu	Lys	Thr	Met	Ala	Gly	Ile	Gln
		115					120					125			
Lys	Val	Glu	Leu	Pro	Glu	Ile	Pro	Asp	Glu	Ala	Lys	Asp	Leu	Thr	Asp
	130					135					140				
Leu	Val	Glu	Ser	Glu	Tyr	Pro	Phe	Ala	Ile	Asp	Pro	Met	Pro	Asn	Leu
145					150					155					160
Tyr	Phe	Thr	Arg	Asp	Pro	Phe	Ala	Thr	Ile	Gly	Asn	Ala	Val	Ser	Leu
				165					170					175	
Asn	His	Met	Phe	Ala	Asp	Thr	Arg	Asn	Arg	Glu	Thr	Leu	Tyr	Gly	Lys
			180					185					190		
Tyr	Ile	Phe	Lys	Tyr	His	Pro	Ile	Tyr	Gly	Gly	Lys	Val	Asp	Leu	Val
		195					200					205			
Tyr	Asn	Arg	Glu	Glu	Asp	Thr	Arg	Ile	Glu	Gly	Gly	Asp	Glu	Leu	Val
	210					215					220				
Leu	Ser	Lys	Asp	Val	Leu	Ala	Val	Gly	Ile	Ser	Gln	Arg	Thr	Asp	Ala
225					230					235					240
Ala	Ser	Ile	Glu	Lys	Leu	Leu	Val	Asn	Ile	Phe	Lys	Lys	Asn	Val	Gly
				245				250						255	

Phe Lys Lys Val Leu Ala Phe Glu Phe Ala Asn Asn Arg Lys Phe Met
 260 265 270
 His Leu Asp Thr Val Phe Thr Met Val Asp Tyr Asp Lys Phe Thr Ile
 275 280 285
 His Pro Glu Ile Glu Gly Asp Leu His Val Tyr Ser Val Thr Tyr Glu
 290 295 300
 Asn Glu Lys Leu Lys Ile Val Glu Glu Lys Gly Asp Leu Ala Glu Leu
 305 310 315 320
 Leu Ala Gln Asn Leu Gly Val Glu Lys Val His Leu Ile Arg Cys Gly
 325 330 335
 Gly Gly Asn Ile Val Ala Ala Ala Arg Glu Gln Trp Asn Asp Gly Ser
 340 345 350
 Asn Thr Leu Thr Ile Ala Pro Gly Val Val Val Val Tyr Asp Arg Asn
 355 360 365
 Thr Val Thr Asn Lys Ile Leu Glu Glu Tyr Gly Leu Arg Leu Ile Lys
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 Ser Met Pro Phe Glu Arg Glu Glu Val
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 35 40 45
 Leu Lys Val Ala Arg Gln Glu His Glu Val Phe Val Asn Ile Leu Lys

11

305 310 315 320
 Asp Val Leu Ser Phe Tyr Leu Gly Arg Lys Ile Asp Ile Ile Lys Cys
 325 330 335
 Ala Gly Gly Asp Leu Ile His Gly Ala Arg Glu Gln Trp Asn Asp Gly
 340 345 350
 Ala Asn Val Leu Ala Ile Ala Pro Gly Glu Val Ile Ala Tyr Ser Arg
 355 360 365
 Asn His Val Thr Asn Lys Leu Phe Glu Glu Asn Gly Ile Lys Val His
 370 375 380
 Arg Ile Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly Pro Arg Cys
 385 390 395 400
 Met Ser Met Ser Leu Val Arg Glu Asp Ile
 405 410

<210> 9

<211> 409

<212> PRT

<213> Borrelia afzelii

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 20 25 30
 Thr Pro Phe Ile Met Lys Asn Phe Leu Phe Asp Asp Ile Pro Tyr Leu
 35 40 45
 Glu Val Ala Arg Gln Glu His Glu Val Phe Ala Ser Ile Leu Lys Asn
 50 55 60
 Asn Leu Val Glu Ile Glu Tyr Ile Glu Asp Leu Ile Ser Glu Val Leu
 65 70 75 80
 Val Ser Ser Val Ala Leu Glu Asn Lys Phe Ile Ser Gln Phe Ile Leu
 85 90 95
 Glu Ala Glu Ile Lys Thr Asp Phe Thr Ile Asn Leu Leu Lys Asp Tyr
 100 105 110

Phe	Ser	Ser	Leu	Thr	Ile	Asp	Asn	Met	Ile	Ser	Lys	Met	Ile	Ser	Gly	115	120	125	
Val	Val	Thr	Glu	Glu	Leu	Lys	Asn	Tyr	Thr	Ser	Ser	Leu	Asp	Asp	Leu	130	135	140	
Val	Asn	Gly	Ala	Asn	Leu	Phe	Ile	Ile	Asp	Pro	Met	Pro	Asn	Val	Leu	145	150	155	160
Phe	Thr	Arg	Asp	Pro	Phe	Ala	Ser	Ile	Gly	Asn	Gly	Val	Thr	Ile	Asn	165	170	175	
Lys	Met	Phe	Thr	Lys	Val	Arg	Gln	Arg	Glu	Thr	Ile	Phe	Ala	Glu	Tyr	180	185	190	
Ile	Phe	Lys	Tyr	His	Pro	Val	Tyr	Lys	Glu	Asn	Val	Pro	Ile	Trp	Leu	195	200	205	
Asn	Arg	Trp	Glu	Glu	Ala	Ser	Leu	Glu	Gly	Gly	Asp	Glu	Leu	Val	Leu	210	215	220	
Asn	Lys	Gly	Leu	Leu	Val	Ile	Gly	Ile	Ser	Glu	Arg	Thr	Glu	Ala	Lys	225	230	235	240
Ser	Val	Glu	Lys	Leu	Ala	Ile	Ser	Leu	Phe	Lys	Asn	Lys	Thr	Ser	Phe	245	250	255	
Asp	Thr	Ile	Leu	Ala	Phe	Gln	Ile	Pro	Lys	Asn	Arg	Ser	Tyr	Met	His	260	265	270	
Leu	Asp	Thr	Val	Phe	Thr	Gln	Ile	Asp	Tyr	Ser	Val	Phe	Thr	Ser	Phe	275	280	285	
Thr	Ser	Asp	Asp	Met	Tyr	Phe	Ser	Ile	Tyr	Val	Leu	Thr	Tyr	Asn	Pro	290	295	300	
Ser	Ser	Ser	Lys	Ile	His	Ile	Lys	Lys	Glu	Lys	Ala	Arg	Ile	Lys	Asp	305	310	315	320
Val	Leu	Ser	Phe	Tyr	Leu	Gly	Arg	Lys	Ile	Asp	Ile	Ile	Lys	Cys	Ala	325	330	335	
Gly	Gly	Asp	Leu	Ile	His	Gly	Ala	Arg	Glu	Gln	Trp	Asn	Asp	Gly	Ala	340	345	350	
Asn	Val	Leu	Ala	Ile	Ala	Pro	Gly	Glu	Ile	Ile	Ala	Tyr	Ser	Arg	Asn	355	360	365	

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 Val Ala Arg Glu Glu His Asp Ala Phe Ala Gln Thr Leu Arg Glu Ala

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Thr	Ser	Asp	Glu	Val 85	Lys	Gln	Gln	Phe	Ile 90	Ser	Glu	Phe	Ile	Asp 95	Glu
Ala	Gly	Val	Glu	Ser	Glu	Arg	Leu	Lys 105	Glu	Ala	Leu	Ile	Glu 110	Tyr	Phe
Asn	Ser	Phe 115	Ser	Asp	Asn	Lys	Ala	Met 120	Val	Asp	Lys	Met 125	Met	Ala	Gly
Val	Arg 130	Lys	Glu	Glu	Leu	Lys 135	Asp	Tyr	His	Arg	Glu 140	Ser	Leu	Tyr	Asp
Gln 145	Val	Asn	Asn	Val	Tyr 150	Pro	Phe	Val	Cys	Asp 155	Pro	Met	Pro	Asn	Leu 160
Tyr	Phe	Thr	Arg	Glu 165	Pro	Phe	Ala	Thr	Ile 170	Gly	His	Gly	Ile	Thr 175	Leu
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Lys	Asp	Pro	Glu	Asn	Asp	Gly	Gln	Leu	Leu	Val	Thr	Glu	Glu	Val	Asp

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 325 330 335
 Ile Lys Cys Gly Gly Gly Asp Glu Ile Ile Ala Ala Arg Glu Gln Trp
 340 345 350
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<211> 413

<212> PRT

<213> Bacillus licheniformis

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 Lys Ala Asp Ile Asn Gly Ala Tyr Asp Arg Leu Lys Glu Phe Leu Leu
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 Met Lys Glu Pro Ala Arg Arg Arg Glu Ser Leu Phe Met Arg Tyr Ile
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Gln Ala Glu His Asp Ala Phe Ala Glu Leu Leu Arg Ser Lys Asp Ile
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Glu Val Val Tyr Leu Glu Asp Leu Ala Ala Glu Ala Leu Ile Asn Glu
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 340 345 350
 Thr Leu Ala Ile Ala Pro Gly Glu Val Val Val Tyr Asp Arg Asn Thr
 355 360 365
 Ile Thr Asn Lys Ala Leu Glu Glu Ala Gly Val Lys Leu Asn Tyr Ile
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 35 40 45

Gln Lys Glu His Asp Gln Phe Ala Gln Thr Leu Arg Asp Asn Gly Val
 50 55 60

Glu Val Leu Tyr Leu Glu Asn Leu Ala Ala Glu Ala Ile Asp Ala Gly
 65 70 75 80

Asp Val Lys Glu Ala Phe Leu Asp Lys Met Leu Asn Glu Ser His Ile
 85 90 95

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Asp Tyr Pro Phe Tyr Met Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
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Asp Pro Ala Ala Ser Met Gly Asp Gly Leu Thr Ile Asn Lys Met Thr
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 180 185 190

His His Pro Arg Phe Ala Asn Gln Gly Ala Gln Val Trp Arg Asp Arg
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	260		265		270	
Thr Val Phe Thr Met Ile Asp Tyr Asp Lys Phe Thr Ile His Pro Gly						
	275		280		285	
Ile Gln Gly Ala Gly Gly Met Val Asp Thr Tyr Ile Leu Glu Pro Gly						
	290		295		300	
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Asn Thr Leu Ala Ile Ala Pro Gly Val Val Val Thr Tyr Asp Arg Asn						
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Val Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly Pro Arg Cys Met						
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Ser Met Pro Leu Val Arg Arg Lys Thr						
	405					

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9/96, 1/20, 11/08, A61P 35/00, A61K 38/50 // (C12N
1/20, C12R 1:35)

(74) Agents: **ELDERKIN, Dianne, B.** et al.; Woodcock Wash-
burn LLP, 46th floor, One Liberty Place, Philadelphia, PA
19103 (US).

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09/723,546 28 November 2000 (28.11.2000) **US**

(71) Applicant (for all designated States except US):
PHOENIX PHARMACOLOGICS, INC. [US/US];
115 John Robert Thomas Drive, Exton, PA 19341 (US).

(72) Inventor; and

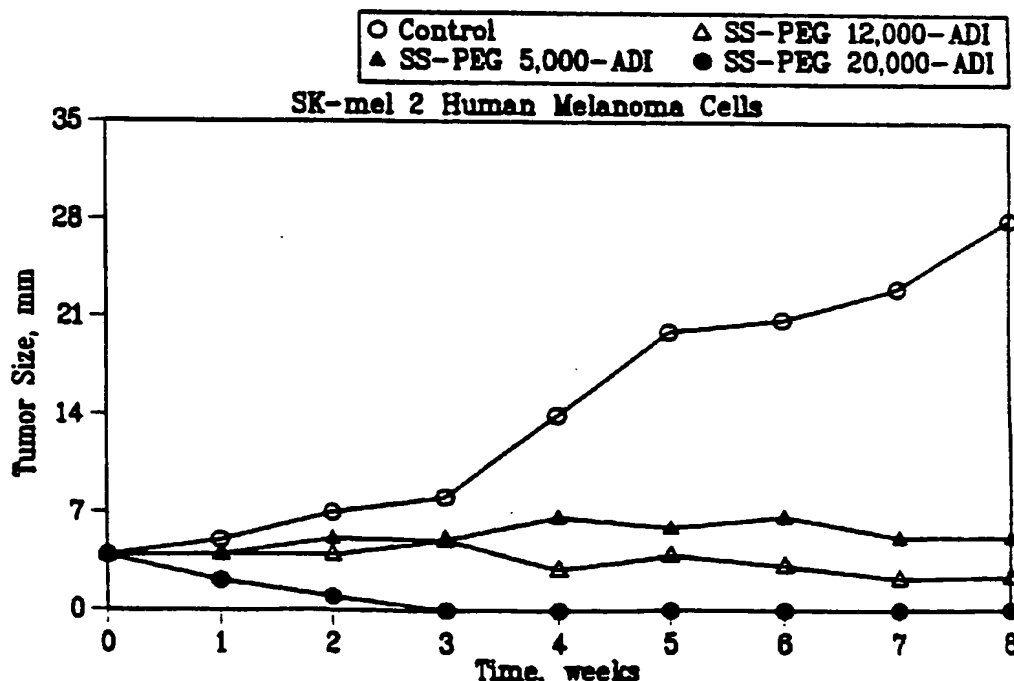
(75) Inventor/Applicant (for US only): **CLARK, Mike, A.** [/];
1276 Scoville Road, Lexington, KY 40502 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

[Continued on next page]

(54) Title: **MODIFIED ARGININE DEIMINASE**



(57) Abstract: The present invention is directed to arginine deiminase modified with polyethylene glycol, to methods of treating cancer, and to methods of treating and/or inhibiting metastasis.

WO 02/044360 A3

**Declarations under Rule 4.17:**

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

- of inventorship (Rule 4.17(iv)) for US only

Published:

- with international search report

(88) Date of publication of the international search report:

19 December 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/29184

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/78 C12N9/96 C12N1/20 C12N11/08 A61P35/00
A61K38/50 //(C12N1/20, C12R1:35)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBL, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 51784 A (PHOENIX PHARMACOLOGICS INC) 19 November 1998 (1998-11-19) the whole document ---	1-37
X	US 6 132 713 A (FIIPULA DAVID RAY ET AL) 17 October 2000 (2000-10-17) examples 6,9 ---	1-37
X	US 5 372 942 A (BUTLER GARY H ET AL) 13 December 1994 (1994-12-13) example 7 --- -/--	1-37

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

3 September 2002

Date of mailing of the international search report

27/09/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Aslund, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/29184

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 200161 Derwent Publications Ltd., London, GB; Class A96, AN 1992-188063 XP002211867 -& JP 04 121187 A (NIPPON MINING CO), 22 April 1992 (1992-04-22) abstract	1-37
X	--- TAKAKU HARUO ET AL: "Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from Mycoplasma arginini." JAPANESE JOURNAL OF CANCER RESEARCH, vol. 84, no. 11, 1993, pages 1195-1200, XP008007607 ISSN: 0910-5050 the whole document	1-37
A	--- DATABASE EMBL 'Online! EBI, Hinxton, UK; 16 December 1997 (1997-12-16) FRASER, C ET AL.: "Borrelia burgdorferi (section 69 of 70) of the complete genome." Database accession no. AE001183 XP002211866 the whole document	9
A	--- KNODLER LEIGH A ET AL: "Cloning and expression of a prokaryotic enzyme, arginine deiminase, from a primitive eukaryote Giardia intestinalis." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 8, 20 February 1998 (1998-02-20), pages 4470-4477, XP002211868 ISSN: 0021-9258 the whole document	11,12
A	--- TAKAKU H ET AL: "Anti-tumor activity of arginine deiminase from mycoplasma arginini and its growth-inhibitory mechanism" JAPANESE JOURNAL OF CANCER RESEARCH, AMSTERDAM, NL, vol. 86, no. 9, 1 September 1995 (1995-09-01), pages 840-846, XP002092819	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US 01/29184

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 27-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-4, 21-24, 27-37 (all partially), 5-6

Arginine deiminase from the genus mycoplasma covalently bonded to polyethylene glycol and uses thereof.

2. Claims: 1-4, 21-24, 27-37 (all partially), 7-8

Arginine deiminase from the genus Streptococcus covalently bonded to polyethylene glycol and uses thereof.

3. Claims: 1-4, 21-24, 27-37 (all partially), 9-10

Arginine deiminase from the genus Borrelia covalently bonded to polyethylene glycol and uses thereof.

4. Claims: 1-4, 21-24, 27-37 (all partially), 11-12

Arginine deiminase from the genus Qiardia covalently bonded to polyethylene glycol and uses thereof.

5. Claims: 1-4, 21-24, 27-37 (all partially), 13-14

Arginine deiminase from the genus Clostridium covalently bonded to polyethylene glycol and uses thereof.

6. Claims: 1-4, 21-24, 27-37 (all partially), 15-16

Arginine deiminase from the genus Enterococcus covalently bonded to polyethylene glycol and uses thereof.

7. Claims: 1-4, 21-24, 27-37 (all partially), 17-18

Arginine deiminase from the genus Lactobacillus covalently bonded to polyethylene glycol and uses thereof.

8. Claims: 1-4, 21-24, 27-37 (all partially), 19-20

Arginine deiminase from the genus Bacillus covalently bonded to polyethylene glycol and uses thereof.

9. Claim : 25

A method for enhancing the plasma half-life of arginine deiminase by covalent bonding to polyethylene glycol.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. Claim : 26

A method for enhancing the tumoricidal activity of arginine deiminase by covalent bonding to polyethylene glycol.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/29184

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